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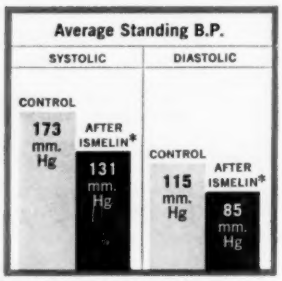
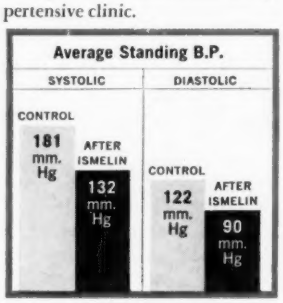
17 of 18 patients (94.4%) treated with Ismelin become normotensive in the erect position. Page and Dustan[1] gave Ismelin orally, alone or in combination with other antihypertensive drugs, to 18 patients daily for 2 to 10 weeks.

RESULTS: All 18 patients had reductions in standing blood pressure; 16 had moderate reductions in supine blood pressure as well. In 17 of the 18 cases, blood pressure levels became normal or near-normal in the erect position.



*During last week of treatment.

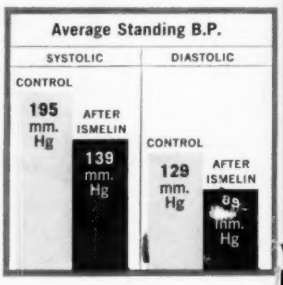
In 14 of 15 patients (93.3%) on Ismelin, blood pressure reduced to normal or near-normal levels in the standing position. Ismelin was administered orally by Frohlich and Freis[2] for 4 to 9 weeks to 15 male patients selected from the hypertensive clinic.



RESULTS: Ismelin evoked a potent antihypertensive response in the erect position: the blood pressure of 14 of the 15 patients dropped to normotensive or near-normotensive levels.

"The response [to Ismelin] was characterized by a potent, orthostatic, antihypertensive effect similar to that seen with the ganglionic blocking drugs but without the side-effects of parasympathetic blockade."[2]

In 15 of 18 subjects (83.3%), guanethidine [Ismelin] reduced high blood pressure to near-normotensive levels. Guanethidine [Ismelin] was administered orally by Richardson and Wyso[3] to 18 male hospitalized patients with hypertension.



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Additional References: 8. Brest, A. N., Duarte, C., Glantz, G., and Moyer, J. H.: Current Therap. Res. *2*:17 (Jan.) 1960. 9. Maxwell, R. A., Mull, R. P., and Plummer, A. J.: Experientia *15*:267 (July 15) 1959. 10. Maxwell, R. A., Plummer, A. J., Schneider, F., Povalski, H., and Daniel, A. I.: J. Pharmacol. & Exper. Therap. *128*:22 (Jan.) 1960. 11. Maxwell, R. A., Plummer, A. J., Schneider, F., Povalski, H., and Daniel, A. I.: Pharmacologist *1*:68 (Fall) 1959. 12. Sheppard, H., and Zimmerman, J.: Pharmacologist *1*:69 (Fall) 1959.

near-normal levels in 80 to 90% of cases[1-3]

RESULTS: "All patients showed definite reduction in blood pressure coincident with administration of [Ismelin]. In most of the subjects [15] standing blood pressure could be maintained near normal levels."[3]

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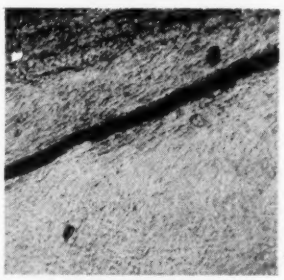

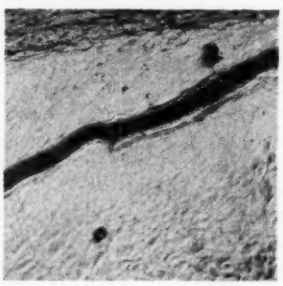

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3.

4.

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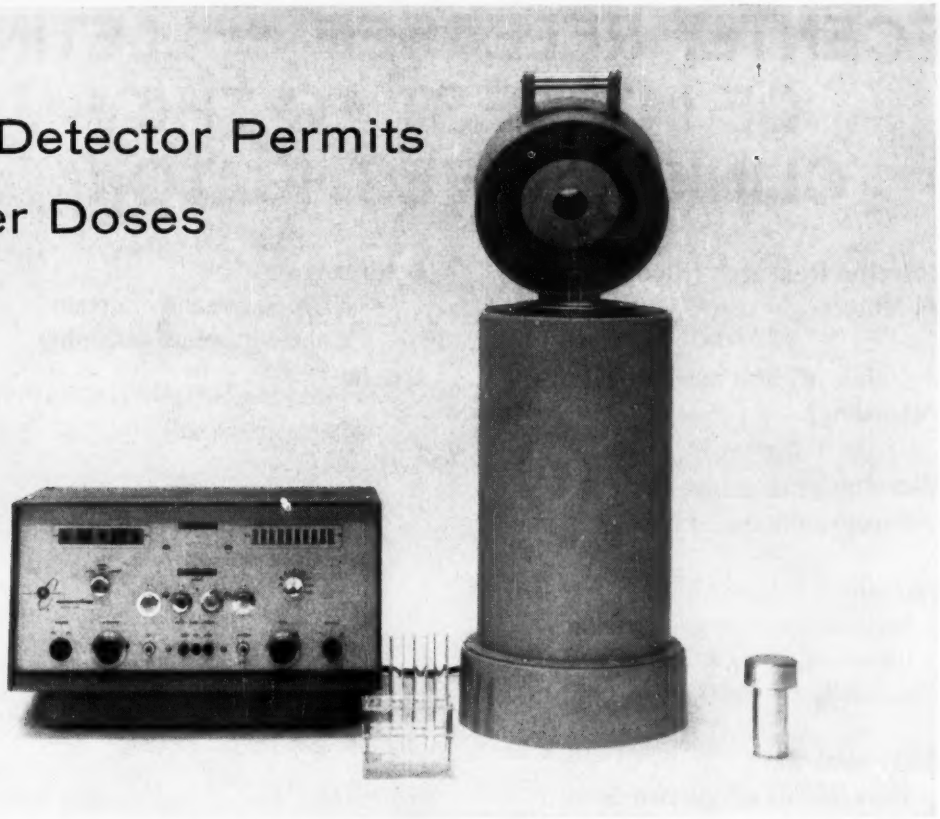

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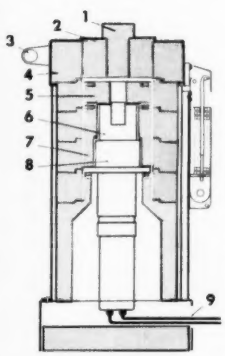

Cross-section of DS-303 Well Detector

1. Removable Plug
2. Removable Plug
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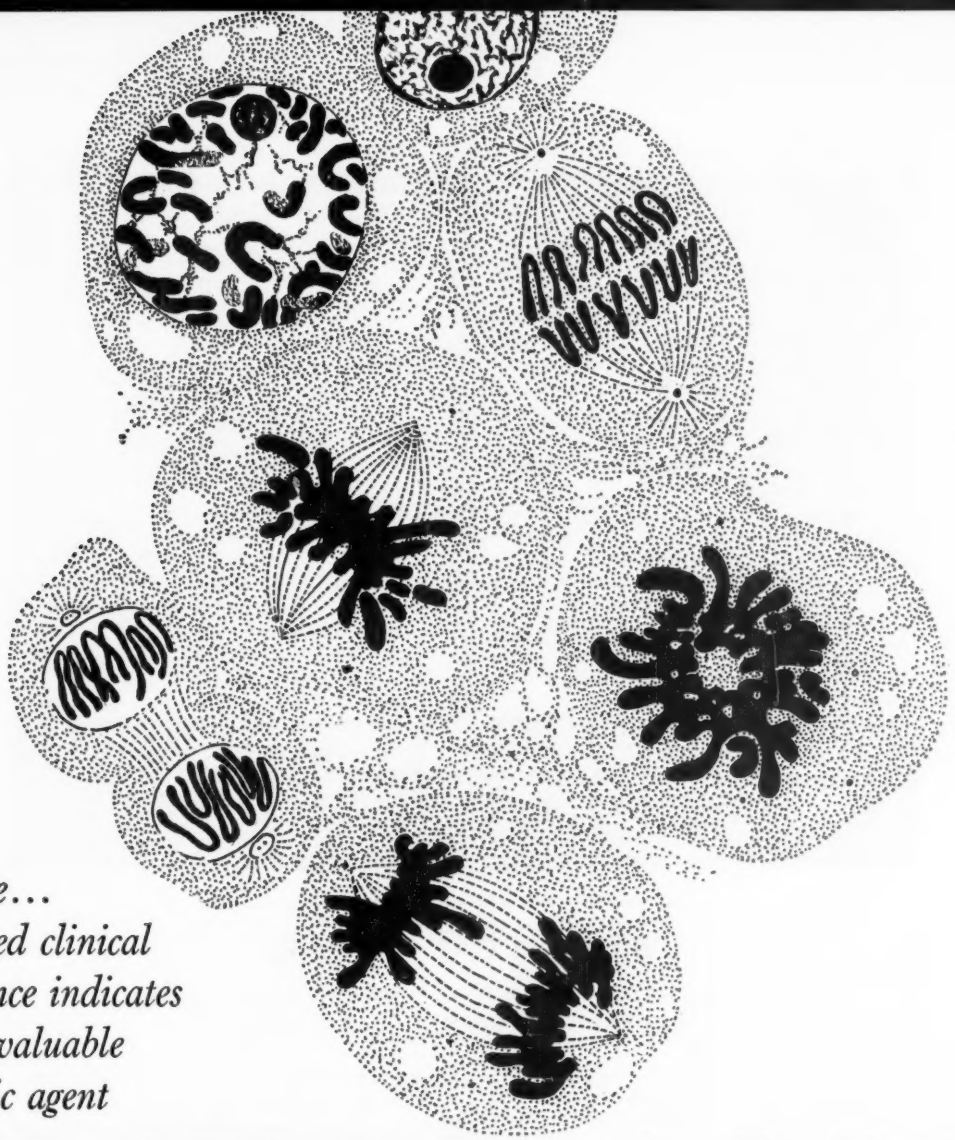

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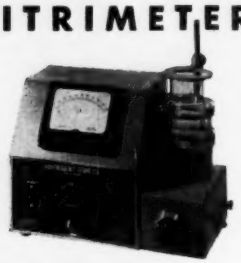

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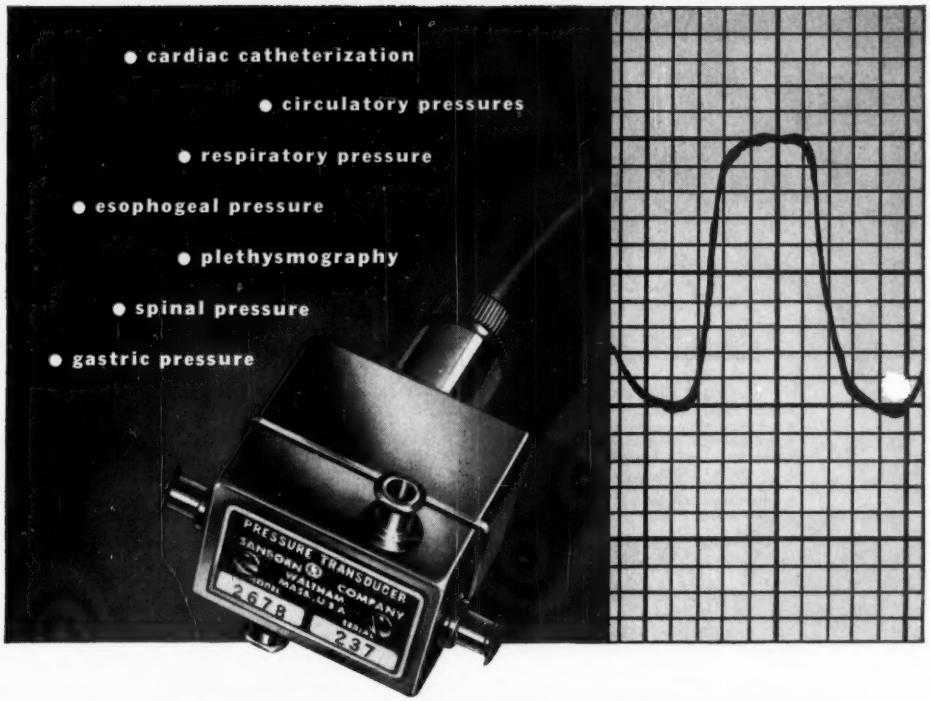

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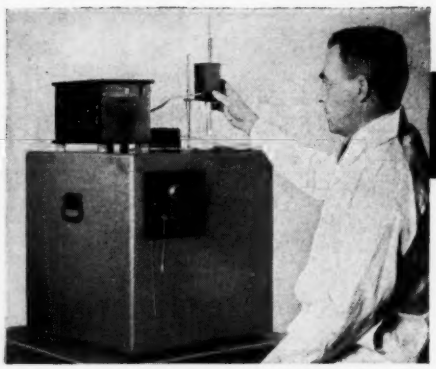

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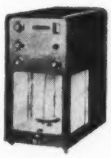

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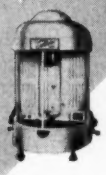

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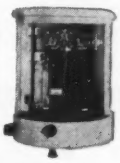

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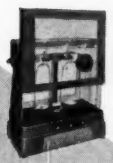

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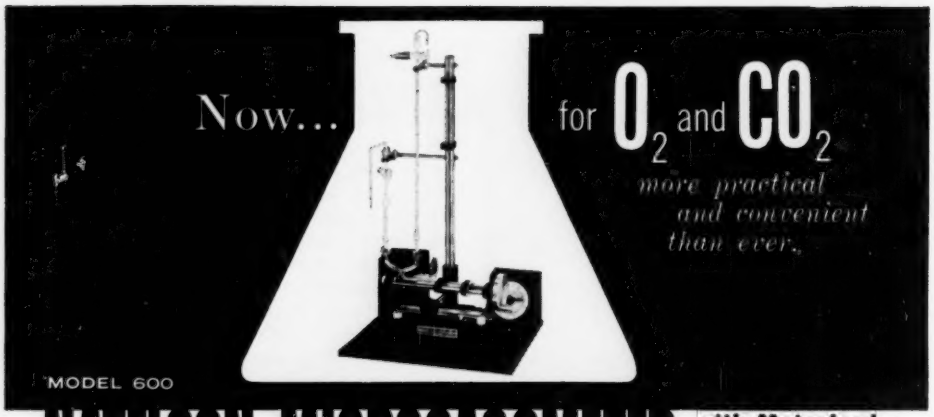

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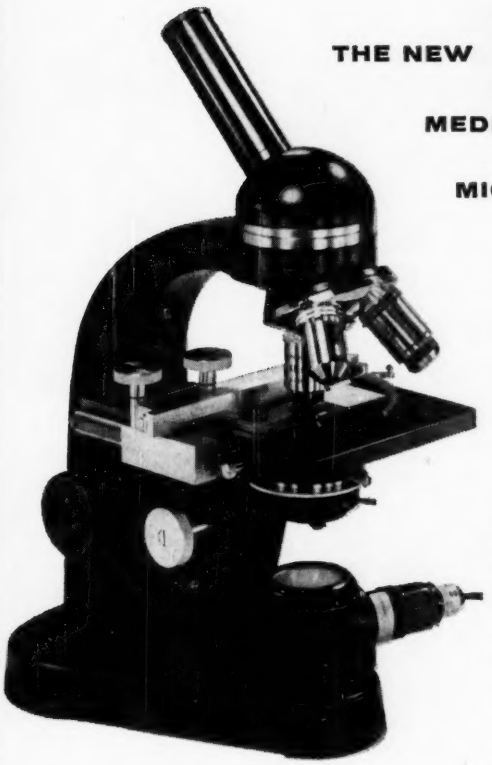

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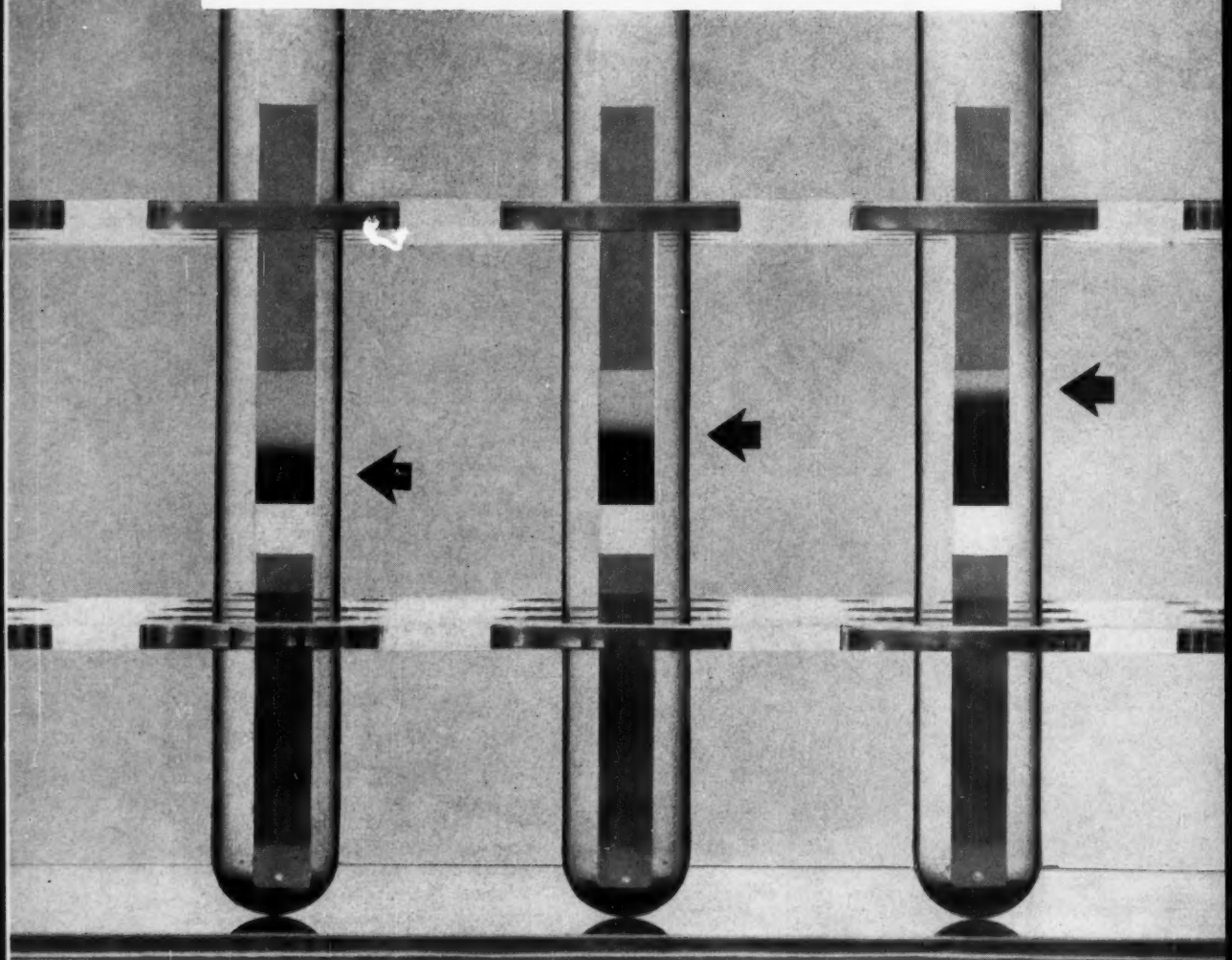

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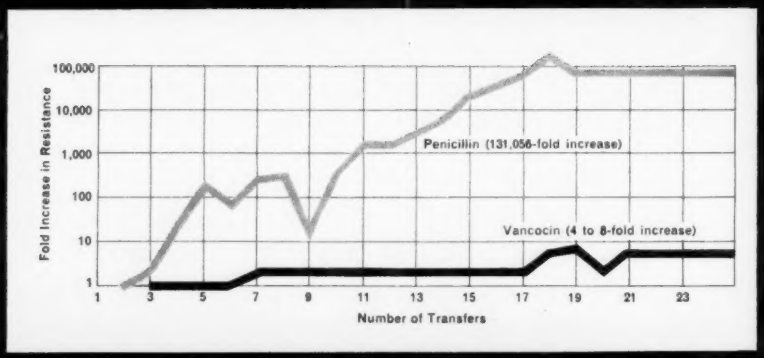

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ABSORPTION OF RADIOACTIVE SODIUM FROM THE INTESTINAL TRACT OF MAN. I. EFFECT OF INTESTINAL MOTILITY.* II. EFFECT OF AN ORGANOMERCURIAL †

By VICTOR W. GROISSER ‡ and JOHN T. FARRAR with the technical assistance of GLORIA C. FERRIS

(From the Medical Service of the Veterans Administration Hospital, and the Department of Medicine, Cornell University Medical College, New York, N. Y.)

(Submitted for publication March 14, 1960; accepted July 15, 1960)

The factors that may influence the kinetics of sodium absorption from the normal and the diseased human small intestine are not well defined. The importance of two such factors, intestinal motility and parenteral administration of organomercurials, was separately investigated in the present study.

The significance of intestinal motility in man has recently been evaluated (3, 4). In normal subjects, administration of methantheline bromide (Banthine) significantly reduced the rate of sodium absorption from the intestinal lumen (3). The diminished transport of sodium was considered to be due to the concomitant reduction in intestinal motor activity. In patients with sprue in relapse, the rate at which sodium entered the circulating blood from the small bowel was also significantly reduced (4, 5). The altered intestinal motor activity observed in this condition, as well as the presence of an enzymatic defect, was thought to play an important role in the abnormal sodium absorption (4).

The effect of mercurials on sodium absorption has been studied in animals. A similarity between the transport mechanisms in the renal tubular and intestinal epithelial cells was suggested by the observation that, when mixed with the saline, and administered into the intestine, various mercurial compounds caused a decrease in the rate of intestinal absorption of the sodium (6) and chloride ions (6, 7).

As a baseline for the present experiments, designed to evaluate the effect of motility and organomercurial administration on sodium absorption, studies were initially performed to determine the characteristics of normal sodium transport, including a measure of variations in normal rates of absorption. The term "absorption" as used in this paper refers to the unidirectional transfer of sodium from the intestinal lumen into the arterial blood.

METHODS

I. Sodium absorption

Forty-five tests of radiosodium absorption were performed on 25 convalescent male hospital patients. All subjects were in normal electrolyte balance and were free of cardiac, hepatic, renal, endocrine and intestinal diseases. Their ages ranged from 25 to 70, with a mean of 43 years.

The observations were made in the morning after an overnight fast of at least 8 hours. The patient remained supine throughout the test. A Miller-Abbott tube was passed under fluoroscopic guidance and its tip positioned in the jejunum approximately 20 to 30 cm beyond the ligament of Treitz. The location of the tube was recorded roentgenographically at the beginning and end of each study. A Cournand needle was inserted into the brachial artery and secured in place. Control samples of blood were obtained. Sodium^{24}, 10 to 15 μc in 30 ml of isotonic saline warmed to 37° C, was introduced into the proximal jejunum at a constant rate during a 60 second period. Sodium^{22}, 7 to 12 μc in 2 ml of isotonic saline, was injected intravenously over a 10 second period, the mid-point of the intravenous injection coinciding with the mid-point of the sodium^{24} administration. During the second minute, the Miller-Abbott tube was flushed with 15 ml of nonradioactive, isotonic saline. The mid-point of the sodium^{24} and sodium^{22} injections was considered to be zero time, and 4 ml of arterial blood was withdrawn at 1-minute intervals for the first 12 minutes, at 3-minute intervals until 30 minutes had elapsed, and then at 5 to 10-minute intervals up to 1 hour.

To determine the degree of reproducibility of the absorption of sodium a second test was performed on 9 patients under identical conditions 1 hour after the control study. In 11 patients, control studies preceded repeat tests performed after intravenous injection of propanthe-

* A portion of this work has been published in abstract form (1).

† Read in part by title at the Fifty-first Annual Meeting, American Society for Clinical Investigation, Atlantic City, N. J., May 4, 1959 (2).

‡ Present address: Seton Hall College of Medicine and Dentistry, Jersey City, N. J.

line bromide (Probanthine). In each repeat test, administration of sodium[24] and sampling of arterial blood were performed exactly as in the control study. However, in order to minimize the contribution of the radioactivity present from the initial test, a much larger dose of sodium[24], 50 to 90 μc, was administered in the same quantity of isotonic saline. The plasma background for purposes of the second test was determined by averaging the radioactivity in two arterial samples drawn immediately prior to the administration of the second dose of sodium[24]. The decrease in this background radioactivity during the first 12 minutes of the repeat test was calculated by extrapolation to be less than 1 to 2 per cent of the total plasma radioactivity at 12 minutes. Absorption was almost complete at 12 minutes in both studies and it is this 12 minute period which is evaluated in greatest detail. The spaces of distribution determined during the control study for each patient were used in calculation of sodium absorption in both the control and repeat tests.

The concentration of isotopic sodium was determined by counting 2 ml of plasma in a conventional well-type scintillation counter. Each sample of plasma containing both sodium[22] and sodium[24] was counted for a period at least long enough to register 10,000 counts. One week later, the sodium[24] ($T_{\frac{1}{2}} = 15$ hours) had decayed to negligible levels, and the plasma was again counted to determine the sodium[22] ($T_{\frac{1}{2}} = 2.6$ years) concentration. Sodium[24] plasma levels on the day of the test were calculated by subtracting the sodium[22] contribution. The dose of sodium[22] and sodium[24] administered was determined by correcting for the total residual radioactivity in the syringes and in the Miller-Abbott tube. Absorption rates were calculated by the formula of Scholer and Code (8) using each patient's observed serial sodium[24] plasma concentration values and sodium[22] spaces of distribution.

Conventional statistical methods were used to determine the 80 percentile range and linear correlation coefficients (R). The probability (p) that a correlation coefficient was obtained by chance was evaluated by analyses of variance and the *t* test (9).

II. Intestinal motility

The motor activity of the small intestine was studied by detection and recording of intraluminal pressure in 21 studies. Two different systems were employed to record these pressure fluctuations. In 11 experiments a pressure-sensitive radio-telemetering capsule was used (10). In 10 tests a small balloon was connected via one lumen of a Miller-Abbott tube to a strain gage manometer (Statham). The balloon was 2 inches long, had a capacity of approximately 45 cc, and during recording was filled with 5 cc of air. Intraluminal pressure was displayed oscilloscopically and permanently recorded by an oscillograph. In all studies, the pressure-detecting device was placed 1 to 1.5 feet distal to the point of administration of sodium[24] into the jejunum. When the radio-telemetering capsule was used, it was held stationary by a thin, peroral thread. When the small balloon was employed, the other lumen of the Miller-Abbott tube was used to administer the sodium[24]-saline solution. The intraluminal pressure records were analyzed for the presence of type I or "segmenting" waves and type III waves (11). For this investigation, type I waves are defined as simple, monophasic pressure waves of 2.5 to 7 seconds in duration. Type III waves are complex waves of longer duration, consisting of type I waves superimposed on an elevation of the baseline of at least 5 cm of H_2O. Determination was made of the percentage of time that type I and type III activity was present by measuring and summating the duration of each wave form. In addition, the average amplitude of the type I waves was calculated.

Jejunal intraluminal pressure was recorded during 15 absorption experiments from the unstimulated small bowel of 11 patients. In 4 of the patients, pressures were recorded during a control and repeat absorption experiment performed under identical conditions. Following the control tests in 6 of the remaining patients, a repeat motility and absorption study was preceded by the intravenous administration of 0.3 mg per kg of propantheline. In these studies, the second sodium[24] absorption-motility test was timed to coincide with the occurrence of a definite anticholinergic effect, i.e., dry mouth, tachycardia or blurring vision.

In 6 additional patients who received propantheline, absorption of sodium[24] was determined after spreading the test saline solution over 2.5 to 3 feet of jejunal mucosal surface. Prior to the administration of propantheline, the tip of the Miller-Abbott tube was advanced to a point 4 to 5 feet distal to the ligament of Treitz. When a peripheral effect of propantheline was observed, mucosal spread of the saline was accomplished by steadily withdrawing a measured length of tubing (2.5 to 3 feet) during the period (1 minute) of continuous sodium[24] administration.

X-ray studies. Preliminary to the radiosodium absorption experiments, radiographic studies were performed to gain information regarding: *1*) the best location for placement of the Miller-Abbott tube, and *2*) the rapidity of passage through the small bowel of a radiopaque solution administered at the same rate and in the same quantity as the sodium[24] test solution and the subsequent flushing isotonic saline solution. Seventeen tests on 13 patients were performed. Thirty ml of sodium 3-acetylamino-2, 4, 6-triiodobenzoate (Urokon Sodium) was administered steadily into the small bowel through a Miller-Abbott tube for 1 minute, followed by a wash of the same dye (15 ml) during the second minute. Radiographs of the abdomen were taken at 1 minute after the midpoint of the initial injection, and then at 2, 5, 10 and 20 minutes.

In the first 6 tests, the tip of the Miller-Abbott tube was placed in the transverse duodenum. In 2 studies a small quantity of dye was noted to reflux into the stomach. Thereafter, to avoid reflux, the tip of the Miller-Abbott tube was placed approximately 20 to 30 cm distal to the ligament of Treitz for the additional 8 X-ray studies and for all of the subsequent sodium experiments.

Examination of the radiographs revealed that at 1 minute the dye was distributed over an area of approximately 1 to 1.5 feet of the jejunum, and at 5 minutes

was dispersed throughout the jejunum and most of the ileum. At 10 minutes some of the dye had entered the proximal colon in several patients. In 3 additional patients, in whom X-ray studies were performed following propantheline administration, the radiopaque dye remained localized in the upper jejunum for a period of at least 20 minutes. Radiopaque marker substances were not used in the sodium^{24} absorption experiments, in order to avoid possible alterations in osmolarity and viscosity of the test saline solution.

III. Mercaptomerin administration

Eleven additional convalescent hospital patients were used in this study. Two tests of sodium^{24} transport from intestinal lumen to arterial blood were performed on each patient. Since only intra-individual differences between arterial appearance curves of sodium^{24} were analyzed, sodium^{22} was not used and spaces of distribution were not determined. Sodium^{24} was administered as in the first portion of the study and arterial blood serially sampled. In addition, a multi-holed urinary catheter was inserted into the bladder of each patient and timed urine collections were made. At the end of each collection period, 100 to 200 cc of air was injected to insure adequate collection. After the initial sodium^{24} absorption study and after at least two 15-minute urine collections, 2 ml of mercaptomerin was given intravenously to each of the 11 patients. A second intestinal absorption test was performed 70 to 120 minutes after the first test and timed to coincide with a marked diuresis, if diuresis occurred. Urinary sodium and chloride were determined by standard methods (12, 13).

Since no correction was made in these studies for variations in the "sodium space," experiments were carried out in 4 other patients to demonstrate that mercaptomerin did not appreciably alter the arterial disappearance curve of intravenously administered sodium. Sodium^{24} was given intravenously prior to mercaptomerin and an arterial disappearance curve plotted. The study was repeated after mercaptomerin and was timed to coincide with a urinary diuresis as in the absorption studies.

RESULTS

I. Characteristics of sodium absorption in normal patients

Rate and total quantity of sodium^{24} absorbed following intrajejunal administration in 22 patients. At 5 minutes a mean total of 61.7 ± 15.3 per cent, and at 12 minutes a mean total of 94.0 ± 11.8 per cent of the administered dose were transferred from intestinal lumen to arterial blood (Figure 1a). Mean absorption during the eleventh minute was 3.9 per cent and during the twelfth minute, 1.8 per cent of the administered dose. By continuing the calculations to 21 minutes in 9 tests, increasingly small increments in the curve of

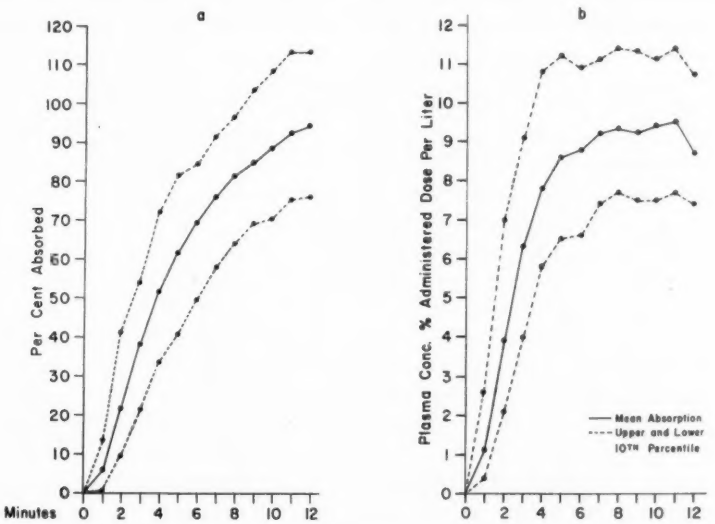

FIG. 1. a. QUANTITY OF SODIUM^{24} ABSORBED FROM THE SMALL INTESTINE OF 22 NORMAL PATIENTS FOLLOWING INTRAJEJUNAL ADMINISTRATION, REPRESENTED AS PER CENT OF ADMINISTERED DOSE. b. RATE OF APPEARANCE OF SODIUM^{24} IN THE ARTERIAL BLOOD. The plasma concentration of sodium^{24} is represented as per cent of administered dose per liter.

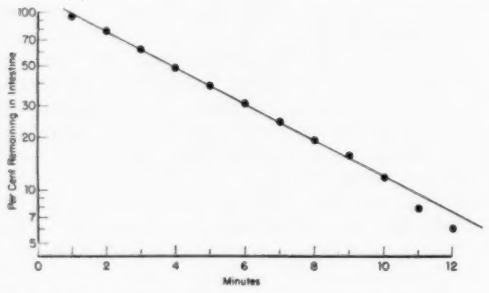

FIG. 2. QUANTITY OF SODIUM^{24} REMAINING IN THE INTESTINE FOLLOWING INTRAJEJUNAL ADMINISTRATION. The rate of absorption is exponential between the second and tenth minute, during which approximately 90 per cent of the total dose is absorbed.

sodium^{24} were noted, approaching or reaching zero absorption. In seven patients absorption of more than 100 per cent of the administered dose occurred at 12 minutes.

The mean rate of absorption of sodium^{24} in terms of the quantity in the intestinal lumen was exponential between the second and tenth minute (Figure 2). During this period, 21 per cent of the amount remaining in the intestine was absorbed per minute.

The initial period during which the plasma concentration curve rose sharply (Figure 1b) corresponded to the period during which the absorption curve also rose rapidly and the largest quantity of sodium^{24} was absorbed (Figure 1a). This might be expected, since the values for absorption of sodium^{24} were derived by integration of the arterial appearance of sodium^{24} and the disappearance of sodium^{22}. The concentration value at 5 minutes was closely correlated ($R = +0.91$) with the average quantity absorbed per minute in the first 5 minutes. During this time more sodium^{24} entered the blood stream than left the intravascular space. At 8 minutes most of the sodium had been absorbed. By 12 minutes the concentration curve of sodium^{24} had already started to decline, while the cumulative absorption curve continued to rise. Thus, little correlation ($R = +0.20$) existed between the concentration value and the total quantity absorbed at 12 minutes.

There was close correlation ($R = +0.96$) between the average quantity absorbed per minute during the first 5 minutes and the time required for the absorption of 50 per cent of the administered dose. This suggests that in normal patients these measures may provide essentially the same information.

Evaluation of a repeat test of sodium absorption under identical conditions in 9 patients. Inspection of mean absorption and concentration curves

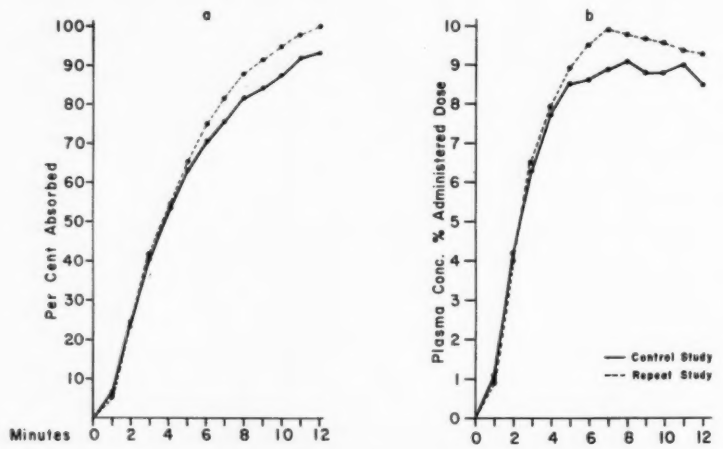

FIG. 3. a. MEAN ABSORPTION CURVE (PER CENT ADMINISTERED DOSE) OF SODIUM^{24} IN CONTROL AND REPEAT CONTROL STUDIES IN NINE NORMAL PATIENTS FOLLOWING INTRAJEJUNAL ADMINISTRATION OF SODIUM^{24}. b. MEAN ARTERIAL CONCENTRATION OF SODIUM^{24} (PER CENT ADMINISTERED DOSE PER LITER) IN CONTROL AND REPEAT CONTROL STUDIES IN NINE NORMAL PATIENTS FOLLOWING INTRAJEJUNAL ADMINISTRATION OF SODIUM^{24}.

TABLE I

Variance analysis between control and repeat sodium absorption tests in 9 patients and single tests in 22 normal patients

	Concentration at 5 min	Absorption, 50% of dose	Absorption rate, first 5 min	Quantity absorbed at 12 min
Variance between single tests in 22 normal patients	3.32	1.56	9.34	140.17
Variance between single tests in 9 normal patients	4.08	1.19	10.81	108.04
Variance between repeat tests in each of the 9 normal patients	2.35	0.81	5.90	43.38

(Figures 3a and 3b) for the control and repeat control test in 9 patients demonstrates the close reproducibility of sodium absorption studies in each patient. No significant differences were found between the two control tests for the following parameters: concentration values at 2, 3, 5 and 8 minutes; and rates of absorption during the first 5 minutes and the 50 per cent absorption time. The total quantity absorbed at 12 minutes was greater in the repeat studies, the difference being statistically significant at the 5 per cent level. This difference at 12 minutes occurred at a time when little sodium was still being absorbed, and large technical errors can be expected. A significantly greater difference was found between single control tests on different patients than between two control tests on the same patient ($p < 0.05$). A variance analysis (Table I) demonstrates the gain in the precision of the experiment when a patient is used as his own control.

II. Relationship between intestinal motor function and absorption of sodium^{24} from the small bowel

Correlation of absorption and motility from the normal small bowel in 11 patients. In the 11 patients in whom sodium^{24} transport and intraluminal pressure studies were measured simultaneously, absorption occurred at a rate similar to that noted for the larger group of 22 patients. A mean of 53 per cent of the administered sodium^{24} was absorbed in 5 minutes and a mean of 89 per cent was absorbed in 12 minutes. Intraluminal pressure recorded during 15 small intestinal absorption experiments varied widely from patient to patient (Table II). There was more intestinal motor activity in the first 5 minutes after administration of the test solution than in the succeeding 7 minutes, possibly due to the motor response of the bowel to the presence of the sodium^{24}-saline solution. The average time during which type I waves were present was 29 per cent of the total 12 minutes (range 2 to 82 per cent). The average amplitude of the type I waves varied for each patient from 0.5 to 14 cm H_2O; mean for the group, 5 cm H_2O. Maximal amplitudes for type I waves for each patient ranged between 2 and 47 cm H_2O. The average duration of type III activity during the 12 minute recording ranged from 0 to 16 per cent; mean, 6 per cent.

Analysis of the data in 15 motility and absorption studies revealed that a positive though not striking correlation existed between percentage of time that type I waves were occurring and the quantities absorbed at 5 minutes ($R = +0.63$) and at 12 minutes ($R = +0.55$), as determined by a comparison of the relative ranking of the motility and absorption values in this series. These correlations are significant at the 2 per cent and 5 per cent levels, respectively. There was no correlation ($R = +0.25$ or less) between either the amplitude of type I waves or the per cent duration of type III waves with the quantity of sodium that was transported from the intestinal lumen into the arterial circulation.

In the three (of four) patients in whom two control tests per subject were performed, an increase or decrease of the amount of type I activity in the repeat control test was always accompanied by a change in absorption in the same direction, although no correlation existed with the degree of change. The remaining patient J.O. (Table II) absorbed the same quantity of sodium at 12 minutes in both control tests, despite a considerable difference in motor activity.

Despite the finding of a moderate positive cor-

relation between absorption and the occurrence of type I activity in the control studies, normal absorption was found to be associated with minimal motor function of any type in several patients (J.O., J.L., W.R., Table II).

Correlation of absorption and motility before and after propantheline administration. Administration of propantheline in eight patients significantly reduced sodium^{24} plasma absorption and concentration values (Figures 4a and 4b) as compared with control levels ($p < 0.05$).

As an indication of a significant change from control levels, the initial slope of the post-anticholinergic plasma concentration curves (Figure 4b) afforded almost as much information as the calculated absorption data (Figure 4a). The degree to which absorption was diminished in individual patients varied considerably and did not correlate well ($R = +0.28$) with the control absorption values (Figure 5). Nor was any correlation observed in these patients between the effect of propantheline on absorption and the severity of the anticholinergic effects such as tachycardia, xerostomia or blurred vision.

Intraluminal pressure was simultaneously recorded during the sodium absorption experiments in six of the eight patients before and after propantheline. Type I waves were abolished or markedly reduced following propantheline administration in four of the six patients. In the

TABLE II

Sodium^{24} absorption and intestinal motility

Patient		Pressure waves during first 5 minutes				Pressure waves during first 12 minutes			
		Quantity absorbed at 5 min	Type I Time present	Type I Av. amp.	Type III Time present	Quantity absorbed at 12 min	Type I Time present	Type I Av. amp.	Type III Time present
		% of dose	*%*	*cc H₂O*	*%*	*% of dose*	*%*	*cc H₂O*	*%*
		I. Two control tests (a and b)							
N.V.	a	68.0	14.1	9.9	3.3	98.5	28.2	11.7	3.8
	b*	75.5				114.2			
H.H.	a	58.0	38.1	5.7	3.3	88.4	31.9	4.4	1.3
	b	69.0	59.8	7.7	18.6	99.3	46.3	6.2	12.0
S.H.	a	55.0	20.4	10.4	15.3	86.5	8.5	4.3	6.3
	b	66.5	36.8	6.7	14.3	85.6	25.9	5.2	5.9
J.O.	a	51.0	40.0	16.0	16.0	92.3	28.1	13.7	16.1
	b	36.0	1.3	2.4	0.0	93.6	3.2	2.5	1.5
H.B.	a	50.0	8.8	3.9	23.0	75.6	5.1	3.2	9.5
	b	60.0	34.5	33.8	2.3	90.2	40.3	3.7	1.0
		II. Control (a) and propantheline (b) tests							
G.P.	a	37.0	23.8	9.3	23.0	68.8	31.2	6.8	11.9
	b	27.5	0.0	0.0	0.0	58.3	0.0	0.0	0.0
A.C.	a	67.0	24.2	11.7	8.3	92.9	26.3	9.9	3.4
	b	40.0	48.5	13.4	0.0	74.3	59.3	20.9	3.6
J.S.	a†	46.5				90.4	81.9	5.8	16.4
	b	9.0				27.6	2.5	0.5	0.0
L.G.	a	67.0	64.6	1.6	0.0	102.7	64.1	1.4	0.0
	b	21.5	34.7	9.6	0.0	62.8	38.0	4.9	0.0
J.L.	a	45.5	4.8	0.9	0.0	81.1	2.0	0.4	0.0
	b	25.5	61.8	9.2	0.0	48.8	46.5	8.0	0.0
W.R.	a	47.0	0.0	1.1	0.0	81.1	0.0	1.1	0.0
	b	26.0	0.0	0.0	0.0	53.9	0.1	2.0	0.0

* Repeat motility study not perormed.
† Twelve-minute motility record technically suitable for only 7 minutes.

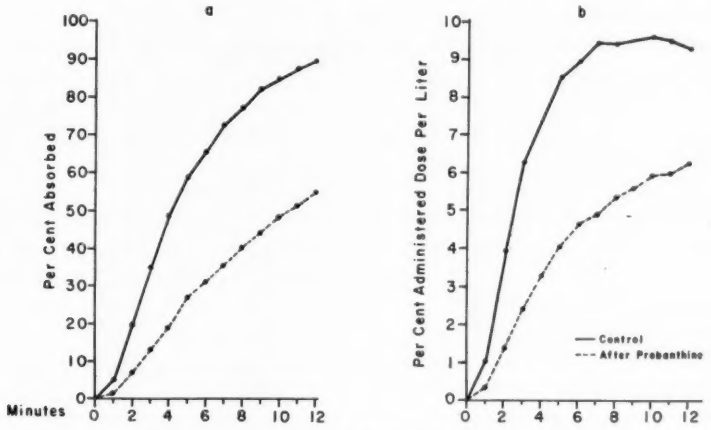

FIG. 4. a. MEAN ABSORPTION CURVES OF SODIUM[24] IN EIGHT NORMAL PATIENTS BEFORE AND AFTER PROBANTHINE ADMINISTRATION. b. MEAN ARTERIAL CONCENTRATION OF SODIUM[24] IN EIGHT NORMAL PATIENTS BEFORE AND AFTER PROBANTHINE ADMINISTRATION.

two remaining patients the total type I motor activity of the jejunum was significantly, although transiently, accentuated by the drug. Serial rhythmic, 11 minute, type I waves were noted for short periods before complete inhibition occurred. Effect of propantheline on type III activity could not be evaluated in three of the patients, since it was absent before the drug as well as after. Propantheline did abolish type III waves, however, in the studies on two patients in whom they were present in the control study.

There was a reduction of both absorption and motor activity following propantheline administration in four patients, but no obvious correlation was noted between the degree to which each of these functions was depressed. In the two pa-

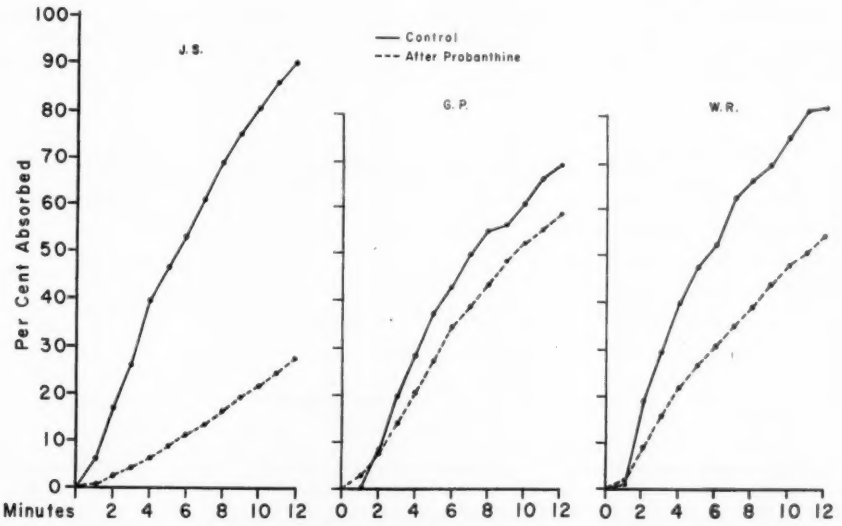

FIG. 5. ABSORPTION OF SODIUM[24] IN THREE NORMAL PATIENTS BEFORE AND AFTER PROBANTHINE ADMINISTRATION. There is no relationship between control levels of sodium[24] absorption and the degree to which Probanthine depresses absorption.

TABLE III

Renal effect of mercaptomerin on urinary flow, and sodium and chloride excretion at beginning of repeat sodium^{24} study*

Type of study	Patient	Urinary changes†		
		Urinary *volume* change in minute flow	*Sodium* change in minute rate of excretion	*Chloride* change in minute rate of excretion
		%	%	%
Sodium^{24} absorption study (intrajejunal administration)	C.S.	287	584	230
	C.H.	33	99	110
	R.A.	458	133	975
	R.M.	36	40	23
	J.S.	848	700	653
	J.M.	414	552	349
	J.J.	112	227	190
	S.C.	300	162	206
	H.G.	936	1,354	1,555
	T.D.	636	299	368
	F.K.	29	456	416
	Mean	372	528	461
	SE	±98	±137	±136
	SD	±324	±455	±449
Sodium^{24} distribution study (i.v. administration)	J.G.	730	256	390
	W.N.	450	602	403
	J.J.	388	869	494
	G.J.	1,017	1,163	961

* Administered intravenously following control sodium^{24} study. Second study started at time of urinary changes recorded in table, 70 to 120 minutes after mercaptomerin administration.

† Per cent change from control levels. All values represent an increase above control levels.

tients who manifested increased motor function, absorption was reduced despite the increase in per cent duration and amplitude of type I activity. It is of considerable interest, however, that in one of these patients (A.C.) sodium^{24} absorption after propantheline was within the 80 percentile range for normal subjects. The other patient (J.L.), who had increased motility after propantheline, had greatly decreased absorption.

Effect of spreading sodium^{24} over jejunal mucosa on propantheline-induced inhibition of absorption. The mean absorption of sodium^{24} fol-

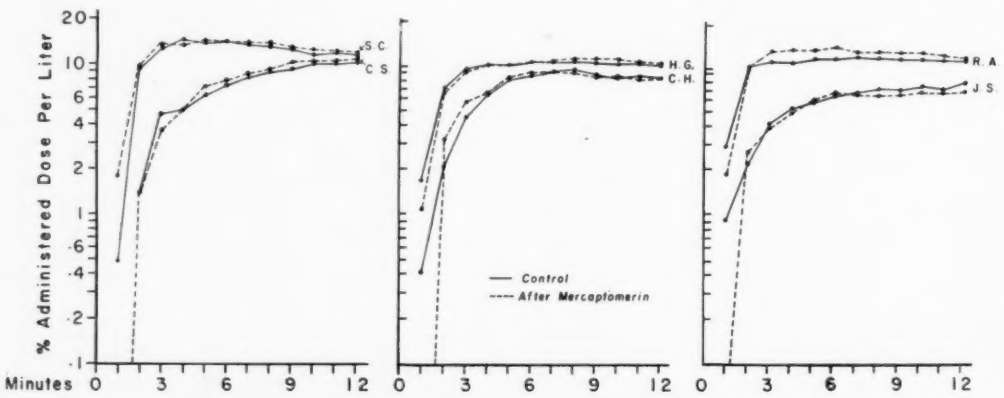

FIG. 6. ARTERIAL CONCENTRATION CURVES AFTER INTRAJEJUNAL ADMINISTRATION OF SODIUM^{24} IN SIX PATIENTS BEFORE AND AFTER MERCAPTOMERIN. These six patients were selected to demonstrate the variation in control absorption which exists between different patients, as well as to demonstrate the absence of a mercaptomerin effect on sodium transport from the intestine.

lowing propantheline administration was not appreciably changed by mechanically spreading the sodium^{24} over approximately 2.5 to 3 feet of jejunal mucosal surface. In the six patients in whom the sodium^{24} was artificially dispersed over the jejunum, absorption at 5 and 12 minutes was 29 and 58 per cent of the administered dose, respectively, compared with absorption of 27 and 55 per cent in the eight patients who received propantheline, but in whom no attempt was made at dispersion of the test solution.

III. Effect of parenteral mercaptomerin administration on sodium absorption

In seven of the 11 patients, a definite, marked increase in natriuresis followed mercaptomerin administration (Table III). In three patients (J.J., R.A. and C.H.) the effect was present but not marked, and in one patient (R.M.) little effect was noted. The mean per cent increase in urinary flow for the 11 patients as compared with control rates of flow was 372 per cent per minute, SE ± 98; the mean per cent increase of sodium excretion was 528, SE ± 137; the mean per cent increase of chloride excretion was 461, SE ± 136.

The arterial plasma concentration curve of sodium^{24} following mercaptomerin administration was in each case very similar to that of the control (Figure 6). The mean differences between the concentrations in the control test, minus those after mercaptomerin at 2, 3, 5 and 8 minutes, respectively, were: + 0.01, − 0.15, − 0.12, + 0.34. These values do not differ significantly from 0, 0, 0, 0 nor do they differ significantly from one another by Hotelling's t test or the conventional F test.

The difference in the control arterial appearance curves of sodium^{24} noted between different patients was very much greater than that noted between the control and post-mercaptomerin tests in the same patient.

The arterial disappearance curve of intravenous sodium^{24} in four other patients was unaffected by the administration of mercaptomerin (Figure 7).

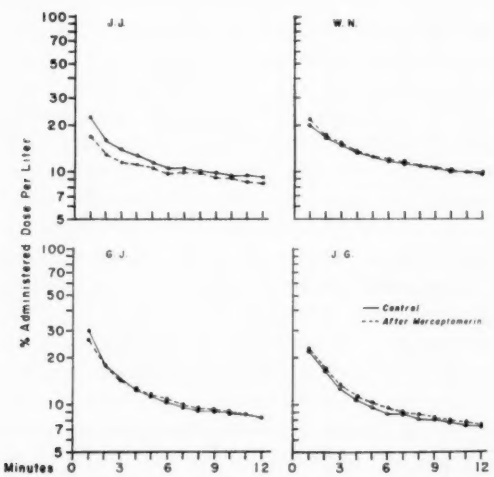

FIG. 7. ARTERIAL DISAPPEARANCE CURVES AFTER INTRAVENOUS ADMINISTRATION OF SODIUM^{24} BEFORE AND AFTER MERCAPTOMERIN. The closeness of the two curves suggests that the mercaptomerin does not significantly affect the distribution of the sodium^{24} during the first 12 minutes.

DISCUSSION

I. Sodium absorption

Sodium^{24} in an isotonic saline solution was transported rapidly from the intestinal lumen into the arterial circulation. In 22 control tests, the unidirectional transfer of 50 per cent of the test dose of isotopic sodium occurred in a mean of 4.3 minutes as compared with a mean of 6.1 minutes in 13 normal patients studied elsewhere (3, 4, 14). Although it is possible that the absorption of greater than 100 per cent of administered sodium^{24} in seven patients represented re-excretion of sodium^{24} into the intestinal lumen, it is more likely that the errors of the method largely accounted for this finding. Reproducibility of the rate at which sodium was absorbed from the intestine in nine repeat control tests was demonstrated to be quite good. On the basis of this reproducibility, the technique of using a patient as his own control was utilized in further studies. When only intra-individual differences were compared, as in the case of the evaluation of the effect of organomercurial administration on the absorption of sodium ions, sodium spaces of distribution were not required. This facilitated the study by eliminating the laborious mathematical calculations needed to integrate concentration with distribution values. It was first determined, however, that no change in the distribution of sodium resulted from mer-

captomerin administration. When inter-individual differences in sodium absorption were analyzed, as in the study of intestinal motility and sodium absorption, it was essential to measure the rate of arterial appearance and disappearance of the test substance. The ability to accurately trace the distribution of sodium throughout the body (15) is essential to this technique, and offers a dimension in the study of absorption from the intact human small bowel which is lacking when rates of absorption of such commonly used substances as glucose, vitamin A, triolein and oleic acid are determined (16). It should be noted, however, that the validity of this method (8, 17) depends in part upon how accurately the distribution of intravenous sodium^{22} reflects the distribution of the sodium^{24} absorbed from the intestine. Since sodium^{24} must pass through the intestinal mucosa, liver and/or lymphatics before reaching the systemic circulation, it is possible that the two isotopes are not identically distributed during the first few minutes. This question has not been completely resolved but experiments in dogs (18) in which sodium^{24} was injected into the portal vein suggest that the difference in route of administration may not introduce significant errors.

II. Intestinal motility and sodium absorption

Moderate positive correlation between type I waves, the "segmenting" waves, and transfer of sodium ions from intestinal lumen to arterial blood was observed. Particularly in patients in whom two control absorption and motility studies were performed, there was a parallel relationship between increased or decreased absorption and corresponding increase or decrease in segmental motor activity. However, the finding of normal rates of sodium transport in the presence of little intestinal motor activity of any type suggests that motility may not be as crucial a factor in the absorption of sodium from a saline medium as has been postulated (3, 4). It is quite possible that intestinal motor activity may be of greater importance to the efflux of sodium from the lumen when the administered sodium is mixed with a more viscous substance such as food. A greater degree of churning would be then required to achieve continuous optimal mucosal contact. The observation that a constant proportion of sodium^{24} remaining in the intestinal lumen enters the blood stream every minute until 90 per cent is absorbed suggests that an optimal absorbing surface is achieved within a few minutes in the normal bowel. A similar exponential rate of absorption has been noted previously for D_2O (18).

Any discussion of the relationship between absorption and motility must include a consideration of the methods used to record motility. In these studies we have employed a device which detects the intraluminal pressure at one point in the jejunum. The intraluminal pressure record at one point may not be the same as that in the remainder of the small intestine, although it is reasonable to suppose that it is. The system we have used does not yield information on gradients of pressure between two adjacent areas of the bowel. Since intraluminal contents move as a result of these gradients, such information would be valuable in assessing those waves that effect propulsion of contents. Ideally intraluminal pressure at several points should be measured and propulsion of contents simultaneously recorded by a separate means. In spite of the limitations of the method used in these studies, it does represent a quantitative, reliable way of recording the motor activity of one segment.

Although the present data confirm the fact that the decrease in absorption which follows administration of an anticholinergic drug may be in part related to diminished motor activity [particularly propulsive activity (3)], a finding of similarly reduced transport of sodium ions after sodium was mechanically spread over 3 feet of jejunal surface suggests that other factors must be considered. It is known that anticholinergics alter secretion into the intestine (19). Atropine has been shown to decrease mean central venous pressure and alter cardiac output (20). It is possible, therefore, that intestinal blood flow is altered by atropine or synthetic parasympathomimetic drugs. Although evidence is lacking, another possibility is that anticholinergics may influence the metabolic activity of the intestinal mucosa which has been demonstrated to cause active sodium and chloride transport (21, 22). The effect of propantheline on the hepatic clearance of sodium is an additional unknown consideration which may be significant.

III. Effect of mercaptomerin on sodium absorption

The present studies indicate that a parenteral organomercurial compound, mercaptomerin, does not appreciably affect the unidirectional transport of sodium^{24} ions during a period when a definite renal effect is manifest. This would suggest that the jejunal and ileal mucosa in man does not respond similarly to the renal tubules after a mercurial diuretic. This is in contrast to previous findings of decreased absorption of sodium (*6, 7*) and chloride (*7*) after mixing the saline with meralluride (Mercuhydrin). The difference in results between these experiments may be a function of the effective concentration of the mercurial at the mucosa. Blickenstaff (*7*) administered 2 ml of meralluride with the NaCl into a jejunal ring fistula in dogs. The resultant concentration of the mercurial compound at the mucosa was probably very greatly in excess of that achieved by parenteral administration of the same dose of mercaptomerin in man. Few data exist on the concentration of mercury in the intestine after parenteral organomercurial administration, and what information does exist is principally derived from animal studies. In the rat and dog, variable amounts of radioactivity were found in the gut wall after parenteral administration of Hg^{203}-labeled chloromerodin [Neohydrin (*23*)]. In these experiments, however, the intestine accounted for a very small fraction of the radioactivity found in the kidney. In one patient with advanced renal disease, autopsied 24 hours after administration of Hg^{203}-labeled mercaptomerin, appreciable radioactivity was present in the colon (*24*), but the small intestine was not studied.

The probable low concentration of a mercurial agent in the small intestinal mucosa of man would raise the question as to whether the failure to inhibit sodium^{24} absorption in these experiments is due, not to differences in the mechanism of sodium transport between intestine and renal tubules but, simply, to the concentration of the mercurial agent in the mucosa.

The possibility exists that a parenteral mercurial might affect intestinal mucosal transport of Na^+ or Cl^- ions at a time other than that chosen in these experiments. It seemed reasonable, however, to use the response of the kidney as an indication of mercurial action.

SUMMARY

1. The quantitative characteristics of normal sodium absorption, i.e., unidirectional transfer of sodium ions from lumen to blood, have been studied by integrating the arterial appearance of sodium^{24} after jejunal administration with the arterial disappearance of sodium^{22} following intravenous administration. In *22* control tests a mean of 62 per cent of the administered dose was absorbed in 5 minutes; a mean of 93 per cent was absorbed in 12 minutes. Arterial concentration curves of sodium^{24} during the first 8 minutes were closely correlated with calculated absorption curves during this period.

2. When calculated in terms of amount of sodium^{24} remaining in the intestinal lumen, transfer of sodium^{24} to arterial blood proceeded at an exponential rate.

3. In nine patients, repeat control tests of intestinal absorption of sodium^{24} under identical conditions indicated a high degree of reproducibility. The technique of using a patient as his own control has been useful in the present studies on the effect of motility and organomercurial administration upon the transport of sodium from the intestinal lumen to arterial blood.

4. Intestinal motility as reflected by jejunal intraluminal pressure was simultaneously recorded in 15 control tests of sodium^{24} absorption. There was some correlation between the amount absorbed and the amount of motor activity present. However, normal absorption occurred in several patients at a time when little motor activity was present. It is concluded that intestinal motility as here recorded probably does not play a critical role in the absorption of isotopic sodium from an isotonic saline solution in the normal small bowel.

5. The unidirectional transfer of sodium from intestinal lumen to arterial blood was significantly decreased after propantheline administration in eight patients. This reduction in absorption was not prevented in six additional patients who received propantheline and had artificial spread of the sodium^{24} over 3 feet of jejunal mucosal surface. These findings suggest that the inhibition of intestinal motility which occurs after an administration of an anticholinergic agent may not be primarily responsible for the decrease in absorption.

6. The effect of an organomercurial, mercaptomerin, on the intestinal absorption of sodium^{24} has been studied in 11 convalescent patients. No effect on the unidirectional transport of sodium from the intestinal lumen to arterial blood was present at a time when a mercurial effect on the kidney was observed.

ACKNOWLEDGMENT

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REFERENCES

1. Groisser, V. W., and Farrar, J. T. Factors influencing the absorption of radioactive sodium (Na^{24}) from the jejunum (abstract). Clin. Res. 1959, 7, 33.
2. Farrar, J. T., and Groisser, V. W. The effect of organomercurials on the absorption of Na^{24} from the human small intestine (abstract). J. clin. Invest. 1959, **38**, 1003.
3. Higgins, J. A., Code, C. F., and Orvis, A. L. The influence of motility on the rate of absorption of sodium and water from the small intestine of healthy persons. Gastroenterology 1956, **31**, 708.
4. Higgins, J. A., Lee, P. R., Scholer, J. F., Reitemeier, R. J., Code, C. F., and Wollaeger, E. E. Absorption of water and sodium from the small intestine of patients with nontropical sprue. J. clin. Invest. 1957, **36**, 265.
5. Newsholme, G. A., and French, J. M. Absorption of ^{24}NaCl from the small intestine in the sprue syndrome. Clin. Sci. 1954, **13**, 607.
6. Visscher, Maurice B. Electrolyte and water movement across the intestinal wall. Ann. N. Y. Acad. Sci. 1953, **57**, 291.
7. Blickenstaff, D. D. Effect of meralluride and of mercuric chloride on intestinal absorption of 0.9% NaCl solution. Amer. J. Physiol. 1954, **178**, 371.
8. Scholer, J. F., and Code, C. F. Rate of absorption of water from stomach and small bowel of human beings. Gastroenterology 1954, **27**, 565.
9. Snedecor, G. W. Statistical Methods Applied to Experiments in Agriculture and Biology, 5th ed. Ames, Iowa State College Press, 1956.
10. Farrar, J. T., and Bernstein, J. S. Recording of intraluminal gastrointestinal pressures by a radiotelemetering capsule. Gastroenterology 1958, **35**, 603.
11. Code, C. F., Hightower, N. C., Jr., and Morlock, C. G. Motility of the alimentary canal in man; review of recent studies. Amer. J. Med. 1952, **13**, 328.
12. Berry, J. W., Chappell, D. G., and Barnes, R. B. Improved method of flame photometry. Industr. engng chem. (Anal.) 1946, **18**, 19.
13. Van Slyke, D. D., Hiller, A., and Plazin, J. Application of Sendroy's iodometric chloride titration to protein-containing fluids. J. biol. Chem. 1947, **167**, 107.
14. Reitemeier, R. J., Code, C. F., and Orvis, A. L. Comparison of rate of absorption of labeled sodium and water from upper small intestine of healthy human beings. J. appl. Physiol. 1957, **10**, 256.
15. Edelman, I. S., Olney, J. M., James, A. H., Brooks, L., and Moore, F. D. Body composition: Studies in the human being by the dilution principle. Science 1952, **115**, 447.
16. Reitemeier, R. J. Newer physiology of absorption from the intestine. Ann. intern. Med. 1959, **51**, 993.
17. Scholer, J. F., Orvis, A. L., Higgins, J. A., and Code, C. F. Use of dual isotopes in determination of rates of absorption in intact animals. Amer. J. Physiol. 1955, **183**, 659.
18. Code, C. F. Normal rates of absorption of H_2O, Na and K, in man and animals. Symp. on Absorption of Water and Electrolytes from the Gastrointestinal Tract. Chicago, Gastroenterology Research Group, October 31, 1958.
19. Goodman, L. S., and Gilman, A. The Pharmacological Basis of Therapeutics, 2nd ed. New York, Macmillan, 1955, p. 548.
20. Berry, J. N., Thompson, H. K., Jr., Miller, D. E., and McIntosh, H. D. Changes in cardiac output, stroke volume, and central venous pressure induced by atropine in man. Amer. Heart J. 1959, **58**, 204.
21. Curran, P. F., and Solomon, A. K. Ion and water fluxes in the ileum of rats. J. gen. Physiol. 1957, **41**, 143.
22. Gilman, A., and Koelle, E. S. Ion transport in the gut circulation. Circulation 1960, **21**, 948.
23. Borghgraef, R. R. M., and Pitts, R. F. The distribution of chlormerodrin (Neohydrin®) in tissues of the rat and dog. J. clin. Invest. 1956, **35**, 31.
24. Aikawa, J. K., and Fitz, R. H. The distribution of HG^{203}-labeled mercaptomerin in human tissues. J. clin. Invest. 1956, **35**, 775.

ANTI-HEMOPHILIC GLOBULIN LEVELS IN CARRIERS OF HEMOPHILIA A*

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Hemophilia A (anti-hemophilic globulin deficiency) is transmitted as an incompletely recessive trait on the X chromosome. Therefore, the daughter of a hemophiliac must be a carrier. In contrast, the daughter of a carrier, e.g., the sister of a hemophiliac, is a "potential" carrier who has an equal chance of being either a carrier or normal. She doesn't know which she is until she bears a hemophilic son; it requires this event to identify her as a known or "obligatory" carrier.

The dilemma of the potential carrier has prompted many investigators to look for some clotting abnormality which might identify a carrier. Nilsson, Blombäck, Thilen and von Francken (1) have summarized the early studies with nonspecific techniques.

The first quantitative anti-hemophilic globulin (AHG) measurements in carriers were published in 1953 by Graham, McLendon and Brinkhous (2). They found low AHG levels in six of ten carriers of mild hemophilia and thought this was a peculiarity of the allelic mutant of the hemophilic gene responsible for mild hemophilia. In apparent confirmation, Gardikas, Katsiroumbas and Kottas (3) reported normal AHG levels in ten obligatory carriers of severe hemophilia. However, several subsequent groups of workers (4–6) demonstrated diminished levels of AHG in some carriers of both mild and severe hemophilia; and, recently, Nilsson and co-workers (1) reported that the vast majority of carriers have low levels of AHG.

The present paper reports on quantitative measurements of AHG made with the Pool-Robinson AHG assay technique (7) in a large number of obligatory carriers and normal women.

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METHODS

Test plasma. Blood was taken from nonfasting subjects between approximately 9 and 10 a.m. using Monocote-E (Armour and Co.) coated needles and silicone (G.E. Dri-film SC-87) coated syringes. Nine parts of blood were added to one part of 0.1 M sodium citrate anticoagulant in polyethylene tubes and centrifuged in the cold for 10 minutes at 10,000 rpm. The resultant platelet-poor plasma was stored frozen at $-20°$ C in 0.7 ml filled, corked glass vials until tested in the assay approximately 3 hours later.

Standard reference plasma. Blood was taken from the same male subject at 2-week intervals. The plasma was prepared and stored as described above except that the plasma was centrifuged a second time to insure the removal of the platelets. Although fresh reference plasma was used every 2 weeks, the AHG content of the stored plasma did not fall over a 6 week period of storage.

The Pool-Robinson assay technique. Our reagents and technique for the Pool-Robinson assay have been described in detail elsewhere (8). The test plasma was assayed at dilutions of 1, 0.5 and 0.25 per cent and the clotting times converted to per cent AHG from the standard reference plasma curve for that day. The mean value for the three dilutions was taken as the final value. All assays were performed with a blind technique.

Thirty-five obligatory carriers and 30 normal women were studied. The obligatory carriers were *1)* daughters of hemophiliacs, *2)* mothers of two or more hemophilic sons, or *3)* mothers of one hemophilic son who had at least one other close male relative with hemophilia. The normal women were laboratory technicians and secretaries in the medical school without personal or family history of abnormal bleeding.

RESULTS AND DISCUSSION

A. Precision and accuracy. AHG was measured in four plasma samples taken from the subjects in one of two ways—either as duplicate samples (from the same venipuncture site but in separate syringes) on each of two successive days or as single samples on four successive weeks. These data are contained in Tables I and II; the weekly samples have been arranged by week of the men-

TABLE I

AHG in duplicate samples on successive days

Subject	Log per cent AHG			
	Day 1		Day 2	
	Sample 1	Sample 2	Sample 1	Sample 2
Carriers				
D.A.	1.78	1.79	1.83	1.87
L.G.	1.78	1.72	1.72	1.78
K.T.B.	1.63	1.65	1.51	1.48
S.F.	1.60	1.59	1.54	1.60
M.H.	1.64	1.62	1.58	1.56
How.	1.88	1.89	1.91	1.94
E.H.	1.94	1.93	1.85	1.91
R.R.	1.76	1.78	1.86	1.89
K.D.	1.89	1.80	1.75	1.83
J.F.	1.83	1.82	1.72	1.75
J.S.	1.68	1.69	1.67	1.66
C.W.	1.77	1.79	1.79	1.74
M.C.	1.81	1.75	1.81	1.83
A.W.	1.32	1.46	1.34	1.36
M.S.	1.82	1.86	1.77	1.80
E.J.	1.95	1.93	1.80	1.82
Normals				
J.R.	1.82	1.86	1.74	1.82
R.G.	1.83	1.83	1.83	1.86
J.C.	1.86	1.92	2.04	2.01
S.S.	2.00	2.01	1.95	2.06
J.G.	1.76	1.76	1.68	1.68
J.P.	1.72	1.75	1.72	1.68
N.H.	2.06	2.06	2.05	1.99
V.H.	2.01	2.01	1.90	1.94
J.L.	2.00	1.99	2.02	1.98
C.F.	1.97	1.95	1.96	1.91
S.L.	1.99	1.99	1.96	1.93
J.M.	1.79	1.82	1.90	1.89
C.C.	1.96	1.95	1.90	1.90

strual cycle. Log per cent AHG is given and was used in all statistical calculations because log transformation (9) resulted in a more normal frequency distribution of the data.

The methods of variance analysis (9) were used to compare error within days, error between days, and error due to possible variation of AHG during different phases of the menstrual cycle. The variances are given in Table III. As expected, the variances between individuals far exceeded those within or between days for the same individual. The variances between days are significantly greater than those within days ($p < 0.001$). In what part this reflects real differences in an individual's AHG level from day to day and in what part it represents technical differences in the test from day to day is not known.

The variances between days and the variances between weeks are of the same order of magnitude. Weekly sampling throughout the menstrual cycle did not bring out differences greater than those encountered on successive days. Thus, AHG may be measured in cyclic women without concern for variation due to the menstrual cycle.

As the very small variance within days indicates, duplicate determinations on the same day do little to increase the accuracy of AHG estimation over a single determination on one day. Greater accuracy requires single determinations on a number of days.

The accuracy with which a single determination on one day estimates the true level found by making single determinations on an indefinite number of days is a matter of practical importance. Figure 1 shows the 90 per cent confidence limits at various AHG levels for a single determination on one day (outer lines) and for the mean of four single determinations on four days (inner lines). These confidence limits were calculated from our combined data on carriers and normals sampled on four successive weeks.

The meaning of these confidence limits may be made more explicit with an example. If a single determination of AHG is 60 per cent, we may have 90 per cent confidence that the true value that might be found from an indefinite number of

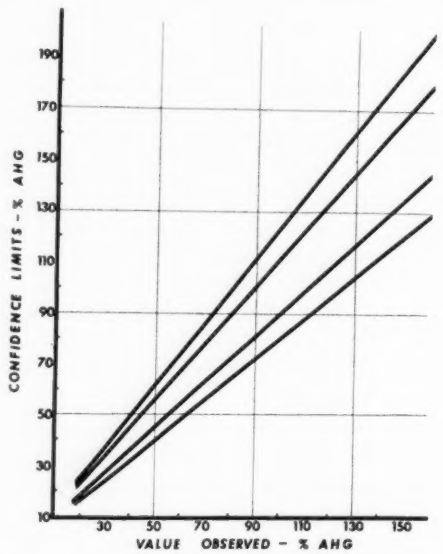

FIG. 1. NINETY PER CENT CONFIDENCE LIMITS OF A SINGLE DETERMINATION OF AHG (OUTER LINES) AND OF THE MEAN OF FOUR SINGLE DETERMINATIONS OF AHG (INNER LINES).

TABLE II

AHG in single samples on successive weeks

Subject	Log per cent AHG			
	Menstrual cycle			
	Days 1–7	Days 8–14	Days 15–21	Days 22–28
Carriers				
L.L.	1.42	1.52	1.58	1.46
S.S.	1.58	1.59	1.78	1.54
M.F.	1.72	1.72	1.65	1.63
B.L.	1.79	1.82	1.73	1.80
L.E.	1.74	1.73	1.81	1.76
L.H.	1.71	1.72	1.76	1.75
O.S.	1.97	2.06	2.06	2.04
E.S.	1.99	1.91	1.90	1.89
A.G.	1.74	1.81	1.80	1.81
T.P.	1.84	1.69	1.88	1.77
Normals				
S.A.	1.94	1.96	2.00	2.00
L.B.	2.00	2.06	2.06	2.06
R.P.	2.01	2.14	1.98	2.18
P.O.C.	1.83	1.99	1.90	1.93
R.L.	1.90	1.86	1.96	1.91
R.G.	2.04	2.16	2.06	2.04
P.S.	1.93	2.04	1.91	1.95
B.D.	2.05	2.09	2.09	2.12
T.W.	2.04	2.14	2.15	2.16
H.B.	1.86	1.92	1.92	1.97
F.S.	2.05	2.09	2.05	2.06
C.M.	2.07	2.03	2.12	2.01
M.J.	2.16	2.15	2.06	2.06
M.L.	1.99	1.80	1.83	1.89

tests over a four week period would have an average value that fell between 48 and 75 per cent AHG. If four single determinations give a mean value of 60 per cent AHG, the 90 per cent confidence limits narrow to between 54 and 67 per cent AHG.

B. Levels of AHG in carriers and in normal subjects. AHG levels for 35 carriers and 30

TABLE III

Summary of variance analysis of data in Tables I and II

Subject	Variation	Sum of squares	Degrees of freedom	Variance
	Duplicate samples on successive days			
Normals	Between individuals	0.5412	12	0.0451
	Within days	0.0199	26	0.0007
	Between days	0.0532	13	0.0041
	Total	0.6143	51	0.0117
Carriers	Between individuals	1.3534	15	0.0902
	Within days	0.0243	32	0.0007
	Between days	0.0787	16	0.0049
	Total	1.4564	63	0.0231
	Single samples on four successive weeks			
Normals	Between individuals	0.3727	13	0.0287
	Between weeks	0.1281	42	0.0030
	Total	0.5008	55	0.0091
Carriers	Between individuals	0.7977	9	0.0887
	Between weeks	0.1023	30	0.0034
	Total	0.9000	39	0.0231

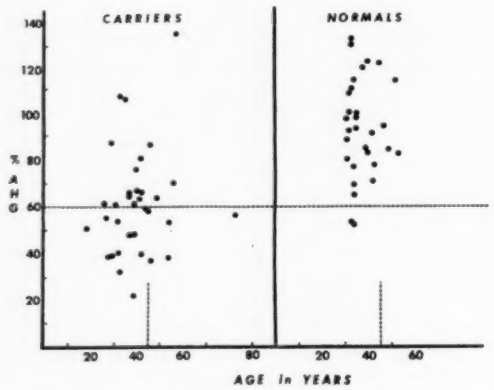

FIG. 2. AHG LEVELS IN CARRIERS AND IN NORMAL SUBJECTS PLOTTED ACCORDING TO AGE IN YEARS. The vertical dotted lines are at age 45 years.

normal women are plotted by age in Figure 2. Except for three subjects from whom only three samples could be obtained, each dot represents the mean of four determinations made from samples taken by either method described above.

AHG levels in normal women varied between 52 and 133 per cent; only 2 of the 30 normal women had AHG levels below 60 per cent of our reference plasma. The carrier range was much wider, between 22 and 135 per cent. Although approximately one-half of the carriers had AHG levels below 60 per cent, many others had levels well within the normal range. AHG did not increase with age in the carrier population; we could not confirm Nilsson and associates' finding (1) of higher AHG levels in carriers over the age of 45 years.

In Figure 3 the values for each group, expressed as log per cent AHG, have been plotted on probability paper against accumulated frequency expressed in per cent. The reasonable linearity of both plots is consistent with sampling from homogenous populations. The wide range of AHG values indicates that many factors in addition to the single gene controlling hemophilia must influence the AHG level in both carriers and normals. Our data do not identify such factors but do exclude age and menstrual cycle.

It can be seen from Figure 3 that 20 carriers per 1,000 may be expected to have AHG levels below about 25 to 30 per cent of normal. This is low enough to predispose to abnormal bleeding. Studies of heterozygous carriers with clinically significant bleeding episodes have been published by Douglas and Cook (10), and by McGovern and Steinberg (11).

The carrier in our series with 22 per cent AHG had bled on the tenth day after tonsillectomy, for three days after one tooth extraction, and for seven days after another. She had developed massive ecchymoses following a minor injury to her leg. Her well documented family history of hemophilia, which followed a sex-linked recessive pattern over several generations, coupled with her normal bleeding time leaves little doubt that she represents another example of significant bleeding in a heterozygous carrier and not of vascular hemophilia.

This carrier comes from a kindred with mild hemophilia (AHG levels of 15 to 20 per cent in affected males). It is of interest that the carriers described by Douglas and Cook (10) came from a family with mild hemophilia. This may also be true of the patient described by McGovern and Steinberg (11). Therefore, although it is well established that low AHG levels may be found in carriers of either mild or severe hemophilia, the question arises as to whether or not there is a rare form of carrier population with *excessively* low AHG levels and clinically significant bleeding episodes in kindreds with mild hemophilia. If our carrier were removed from our series on these

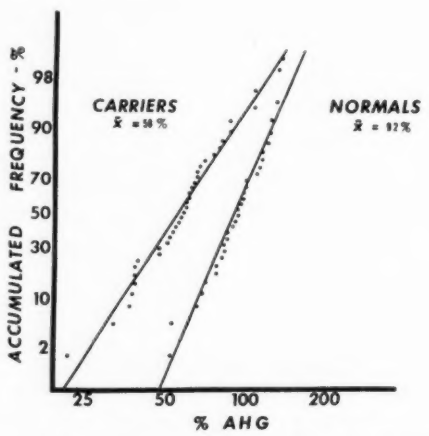

FIG. 3. ACCUMULATED FREQUENCY IN PER CENT PLOTTED ON PROBABILITY PAPER (9) AGAINST OBSERVED VALUE OF AHG IN PER CENT (LOGARITHMIC SCALE). The carriers are identified by circles, the normal subjects by squares.

grounds, then the mean shown in Figure 3 would remain essentially unchanged, but the variances of the carrier and normal groups would become more nearly equal. This would alter slightly the data shown in Figures 4 and 5 (see below). However, in view of the scantiness at present of the evidence for a separate carrier population, we have considered her as an ordinary carrier in our statistical calculations.

Seven of our carriers had AHG levels between 30 and 40 per cent. None had bled abnormally despite the fact that all had had teeth extracted and many had been through major surgery. This is indirect evidence that AHG levels above 30 per cent are usually adequate for hemostasis.

C. Recognition of the carrier by AHG assay. Since the daughter of a carrier has an equal chance of being either normal or a carrier, the accumulated frequencies shown in Figure 3 may be used to calculate her chances of being a *true carrier at or below* any observed AHG level. This is shown in Figure 4. Although each laboratory uses a different 100 per cent reference plasma, the value for the mean of a normal population will probably be found within the limits of 90 to 110 per cent of the particular reference plasma, respectively. Linear interpolation between these curves is probably adequate for practical purposes. Therefore, once the normal mean for a reference plasma has been established, Figure 4 may be used to estimate the probability that a *potential carrier* may be a *true carrier at or below* any observed per cent AHG of that reference plasma.

The mean of our carrier series was 58 per cent AHG. Figure 4 shows that 95 of 100 *potential* carriers with an AHG level *equal to or below* 58 per cent of our reference standard will be *true* carriers. Approximately three-fourths of our carriers had AHG levels below 70 per cent. From line A of Figure 4 we see that a *potential* carrier with an AHG level *at or below* 70 per cent of our reference standard has better than four chances out of five of being a *true* carrier. Thus, it would appear that AHG assay by the thromboplastin generation technique enables one to recognize with reasonable accuracy about 75 per cent of the *true* carriers in a *potential* carrier population.

In a similar fashion it is possible to estimate the probabilities that a *potential* carrier may be *normal* when her AHG level *equals or exceeds* any observed value. This is shown in Figure 5.

As seen from line A of Figure 5, a potential carrier with an AHG level *at or above* 80 per cent of our reference standard has four chances out of

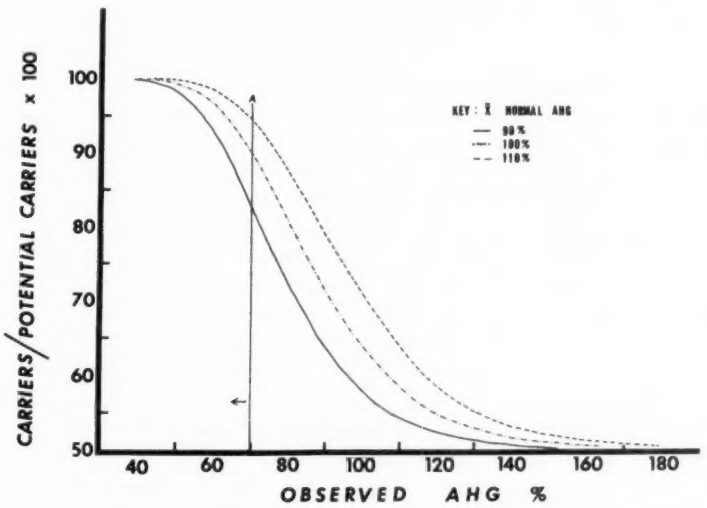

FIG. 4. THE PERCENTAGE OF CARRIERS IN A POTENTIAL CARRIER POPULATION *AT OR BELOW* ANY GIVEN OBSERVED LEVEL OF AHG. Values are plotted for reference plasma which gives a mean for a normal population of 90, 100 and 110 per cent, respectively.

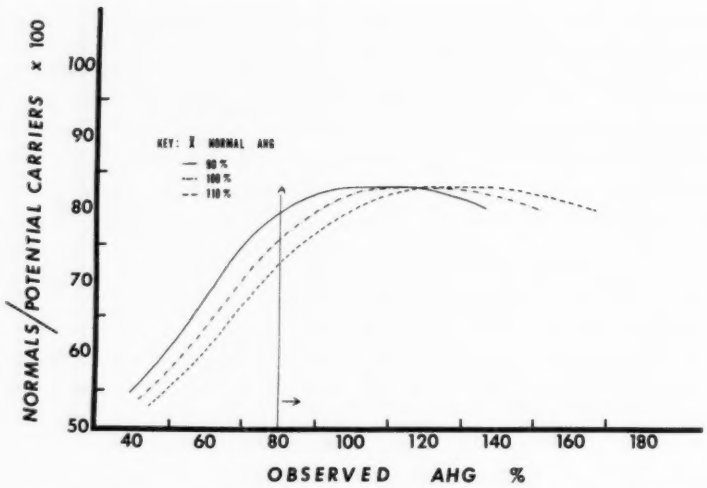

FIG. 5. THE PERCENTAGE OF NORMAL SUBJECTS IN A POTENTIAL CARRIER POPULATION *AT OR ABOVE* ANY GIVEN OBSERVED LEVEL OF AHG. Values are plotted for reference plasma which gives a mean for a normal population of 90, 100 and 110 per cent, respectively.

five of being *normal*. More than two-thirds of the normal members of a potential carrier population would fall into this group. The chances of being normal do not increase significantly *at or above* higher observed AHG values due to the difference in the slope of the two lines in Figure 3. Thus, a *potential* carrier with a high normal AHG level still retains one chance in five of being a *true* carrier.

Our data confirm and extend the observations made with thromboplastin generation techniques of AHG assay in groups of carriers by Pitney and Arnold (6) and by Nilsson and co-workers (1). The latter group also measured AHG in their carriers by a second technique, based upon the correction of the prolonged recalcification time of hemophilic plasma, which resulted in a clearer separation of carriers from normals.

Review of their data discloses *1*) that both techniques yielded the same mean and wide range of AHG levels in normal subjects, and *2*) that, although the majority of carriers had lower values with the recalcification technique, occasional carriers had lower values with the thromboplastin generation technique. These observations raise the possibility that the seemingly more distinct separation by the recalcification time technique may be due to the measurement of a second variable which helps to distinguish carriers from normals. Otherwise, one must postulate that thromboplastin generation techniques of AHG assay give falsely high values in carriers but not in normal subjects. A comparison of these two methods of AHG assay in a variety of conditions in which AHG levels vary appears necessary before this question can be resolved.

SUMMARY

1. Plasma AHG levels were measured in obligatory carriers of hemophilia A and in normal women by the Pool-Robinson assay technique. Four samples were taken by methods which permitted the evaluation of error between duplicates on the same day, between single determinations on successive days, and between single determinations on successive weeks. AHG levels did not vary with the phase of the menstrual cycle.

2. The normal women had a mean AHG value of 92 per cent of our reference plasma (range 52 to 133 per cent). The carriers had a mean AHG value of 58 per cent (range 22 to 135 per cent). Both sets of data appeared to be normally distributed when log per cent AHG was plotted on probability paper.

3. One carrier with 22 per cent AHG had experienced clinically significant bleeding. Seven

carriers with AHG levels between 30 and 40 per cent had not bled abnormally. The distribution of AHG values in our carrier series suggests that AHG levels low enough to predispose to bleeding, i.e., below 30 per cent should occur in about 20 carriers per 1,000.

4. Probability calculations indicate that this AHG assay will detect with reasonable accuracy (four chances out of five) about 75 per cent of the *true* carriers in a *potential* carrier population and about 66 per cent of the *normal members* in a *potential* carrier population. However, a *potential* carrier with even a very high AHG level still retains one chance in five of being a *true* carrier.

REFERENCES

1. Nilsson, I. M., Blombäck, M., Thilen, A., and von Francken, I. Carriers of hemophilia A: A laboratory study. Acta med. scand. 1959, **165**, 357.
2. Graham, J. B., McLendon, W. W., and Brinkhous, K. M. Mild hemophilia: An allelic form of the disease. Amer. J. med. Sci. 1953, **225**, 46.
3. Gardikas, C., Katsiroumbas, P., and Kottas, C. The antihaemophilic-globulin concentration in the plasma of female carriers of haemophilia. Brit. J. Haemat. 1957, **3**, 377.
4. Biggs, R., and Macfarlane, R. G. Haemophilia and related conditions: A survey of 187 cases. Brit. J. Haemat. 1958, **4**, 1.
5. Didisheim, P., Ferguson, J. H., and Lewis, J. H. Hemostatic data in relatives of hemophiliacs A and B; evidence for modifying the classical sex-linked recessive hypothesis. A.M.A. Arch. intern. Med. 1958, **101**, 347.
6. Pitney, W. R., and Arnold, B. J. Plasma antihaemophilic factor (AHF) concentrations in families of patients with haemorrhagic states. Brit. J. Haemat. 1959, **5**, 184.
7. Pool, J. G., and Robinson, J. Assay of plasma antihaemophilic globulin (AHG). Brit. J. Haemat. 1959, **5**, 17.
8. Rapaport, S. I., Ames, S. B., and Mikkelsen, S. The levels of antihemophilic globulin and proaccelerin in fresh and bank blood. Amer. J. clin. Path. 1959, **31**, 297.
9. Moore, F. J., Cramer, F. B., and Knowles, R. G. Statistics for Medical Students. Philadelphia, Blakiston, 1951, pp. 1–113.
10. Douglas, A. S., and Cook, I. A. Deficiency of antihaemophilic globulin in heterozygous haemophilic females. Lancet 1957, **273**, 616.
11. McGovern, J. J., and Steinberg, A. G. Antihemophilic factor deficiency in the female. J. Lab. clin. Med. 1958, **51**, 386.

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THE PULSATILE NATURE OF THE PULMONARY CAPILLARY BLOOD FLOW *

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(Submitted for publication May 23, 1960; accepted July 8, 1960)

The nature of the pulmonary capillary blood flow is fundamental for the understanding both of pulmonary hemodynamics and of respiratory gas exchange. Direct examinations of the surface of the lung in the cat, rabbit, dog and rat (1–5) have indicated that pulmonary capillary blood flow is basically non-pulsatile. On the other hand, experimental studies by Lee and DuBois (6), using the body plethysmograph in man, have suggested that pulmonary capillary blood flow is consistently pulsatile. Their experimental evidence consisted of a rhythmic distortion of the record, synchronous with the heart beat, obtained during breathholding with open glottis, after a single inspiration of nitrous oxide.

However, since the first experiences with the pneumotachograph, it has been recognized that cardiovascular pulsations produce rhythmic changes of pressure in the conducting airways of the lungs. These rhythmic changes in intrapulmonary pressure are detected by the pneumotachograph through the changes in the rate of air flow which they produce. This phenomenon, illustrated in the pneumotachographic record of Figure 1, was recognized by Lilly (7) and designated by him as "cardiac air pulse." Subsequently, Bartlett, Brubach and Specht (8) found that rhythmic changes in the pneumotachographic record could be correlated with the pulsations observed in the plethysmographic pressure record obtained during breathholding after an inhalation of ambient air.

In their original studies on the rate of pulmonary capillary blood flow by the plethysmographic method, Lee and DuBois (6) referred to "mechanical gas compression with the heart beat" to account for oscillations in the plethysmographic pressure record after a breath of ambient air. However, they invoked a completely different mechanism, i.e., the pulsatile uptake of nitrous oxide by blood perfusing the pulmonary capillaries, to account for similar, but larger, oscillations after a breath of nitrous oxide.

The present study was designed to distinguish between the role of pulsatile pulmonary capillary flow and the pulsations of the heart and great vessels in the genesis of the rhythmic distortions of the plethysmographic record.

SUBJECTS AND METHOD

Twenty-two tests were performed on 6 normal subjects. For each test, the subject was seated in an aluminum body plethysmograph which also contained a bag of the test gas. The gas in the bag was either 80 per cent nitrous oxide in oxygen, air, 100 per cent helium, or 100 per cent sulphur hexafluoride. Each subject inspired maxi-

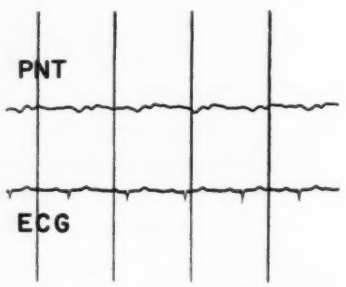

FIG. 1. PNEUMOTACHOGRAPHIC (PNT) AND ELECTROCARDIOGRAPHIC (ECG) TRACINGS OBTAINED FROM A NORMAL SUBJECT DURING BREATHHOLDING WITH THE GLOTTIS OPEN, AFTER A BREATH OF AMBIENT AIR. Rhythmic pulsations, related to the heart beat, are seen in the pneumotachographic record. The upward deflections of the pneumotachographic record represent expiratory flow.

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mally from the bag after a maximum expiration and held his breath with mouth and glottis open for the next 20 to 30 seconds. In some instances the breathholding was also performed after a slight expiration following the maximal inspiration. During breathholding, the pressure in the plethysmograph and the electrocardiogram were recorded. The pressure was measured by an electronic system consisting of a Statham transducer (PL5TC-0.2-350), a SG-A-6 amplifier and an Electronics for Medicine oscilloscopic recording apparatus. The sensitivity of the system was such that the introduction of 100 ml of air into the plethysmograph produced a deflection of 25 mm in the record.

Each of the 6 subjects underwent at least three successive periods of breathholding using nitrous oxide, air and helium as test gases; 2 of the 6 underwent an additional period with sulphur hexafluoride as the inspired gas. Each subject performed this sequence 2 to 6 times. Each time the order of exposure to the different test gases was varied.

In order to relate the pneumotachographic pulsations to changes in intra-airway pressure, the closed system illustrated in Figure 2 was used. The system consisted of a 0.5 L bottle, a pneumotachograph and a rubber mouthpiece connected in series. The test gas was contained in a rubber bag which communicated with the mouthpiece through a two-way valve. By introducing a Statham transducer (P23-2D-250) it was possible to measure simultaneously the instantaneous variations in pressure and flow in this system and to record them with the electrocardiogram. The same respiratory maneuvers which were performed in the body plethysmograph were repeated using this system. The use of a bottle to record airway pressures had two advantages over the plethysmograph: *1*) the pressure variations were amplified considerably, and *2*) the effects of a change in body position on the contour of the pulsations could be assessed.

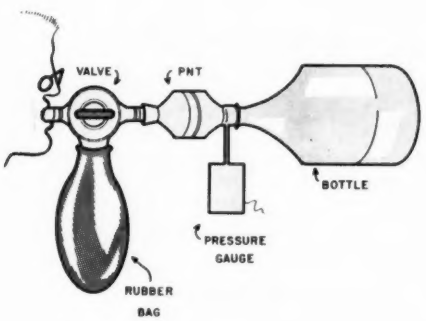

FIG. 2. SYSTEM USED FOR THE DETAILED ANALYSIS OF THE RELATIONSHIP BETWEEN THE CHANGES IN PRESSURE IMPOSED ON THE CONDUCTING AIRWAYS BY THE CARDIOVASCULAR PULSATIONS AND THE PULSATIONS SEEN IN THE PNEUMOTACHOGRAPHIC RECORD. The records obtained using this system are illustrated in Figure 7.

RESULTS

Figures 3, 4, 5 and 6 are records obtained during studies with the body plethysmograph. A typical tracing following a single breath of nitrous oxide appears as the left upper panel of Figure 3. This figure shows the electrocardiogram and the record of the changes of pressure with time during breathholding. It may be seen that: *1*) pressure within the plethysmograph falls gradually during breathholding; *2*) the gradual decline in pressure is rhythmically deformed by upward displacements of the baseline; *3*) the oscillations are synchronous with the heart beats; and *4*) each deflection reaches its peak approximately 0.2 of a second after the R wave of the electrocardiogram. The three other panels of this figure indicate that the rhythmic oscillations persist when air or even nonabsorbable gases are substituted for nitrous oxide. The different slopes with the different test gases are attributable to : *1*) the absorption of test gases such as nitrous oxide, by blood perfusing the pulmonary capillaries, *2*) the progressive warming of the plethysmograph by the patient, and *3*) the respiratory exchange ratio of the subject.

Similar tracings for three other subjects appear in Figure 4. This figure illustrates slight variations in the amplitude of the pulsations from subject to subject, and from gas to gas; for example, in Subjects E.B. and M.R. large oscillations occurred with all three gases; in the other subject, R.M., the oscillations were larger with nitrous oxide.

Figure 5 allows a closer examination of individual pulsations after a breath of nitrous oxide and of their relationships to the gradual decline in pressure. This figure shows that: *1*) the pressure falls in a straight line rather than in a stepwise manner, and *2*) the deflections are positive.

Figure 6 illustrates the effect of reversing the slope of the pressure curve on the nature of the deflections. The slope was reversed by a continuous inflow of oxygen into the plethysmograph after the subject had taken the breath of nitrous oxide. This device, which was used to remove the uncertainty of recognizing upward deflections from a falling baseline, emphasizes the positive nature of the deflections.

Except for minor variations, tracings identical to those of Figures 3, 4 and 5 were obtained from

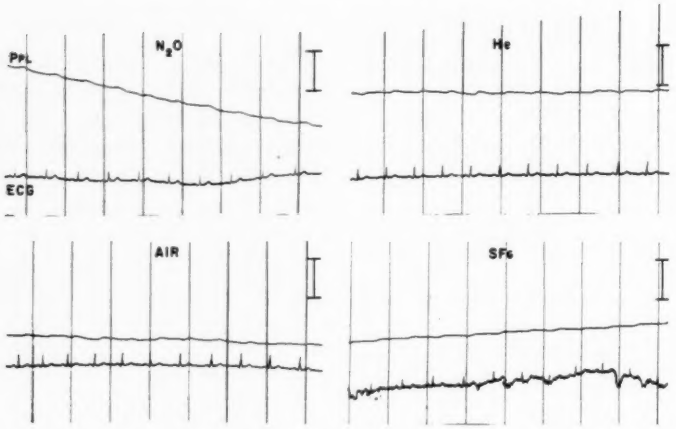

FIG. 3. PLETHYSMOGRAPHIC PRESSURE RECORD (P_{PL}) AND ELECTROCARDIOGRAM (ECG) OBTAINED FROM A NORMAL SUBJECT USING DIFFERENT TEST GASES. The gases were nitrous oxide (N_2O), helium (He), ambient air (air) and sulphur hexafluoride (SF_6). Similar types of rhythmic distortions of the pressure record are seen with all the test gases. The interval between the vertical time lines is 1 second. The vertical distance between brackets equals the deflection produced in the pressure record by the introduction of 100 ml of air into the body plethysmograph. The upward deflections of the pressure record represent an increase in pressure in the body plethysmograph.

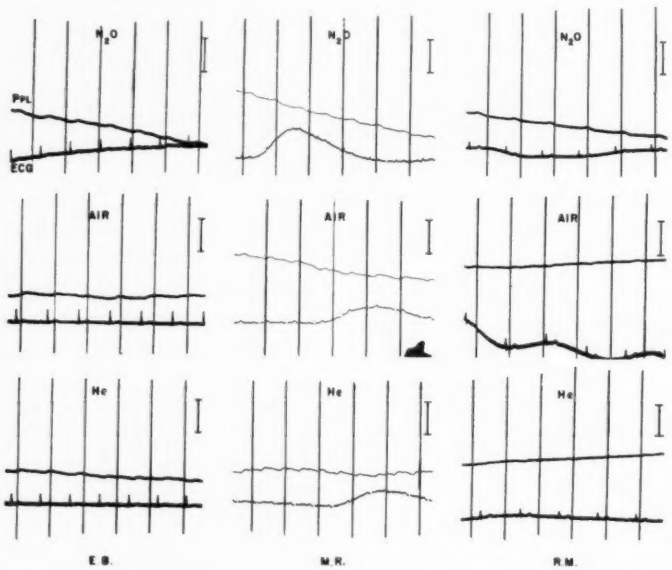

FIG. 4. PLETHYSMOGRAPHIC PRESSURE RECORDS (P_{PL}) AND ELECTROCARDIOGRAM (ECG) OF THREE NORMAL SUBJECTS. This figure illustrates the slight variations which are encountered in records from normal subjects using the different test gases. The time lines and the vertical distance between brackets are the same as those in Figure 3.

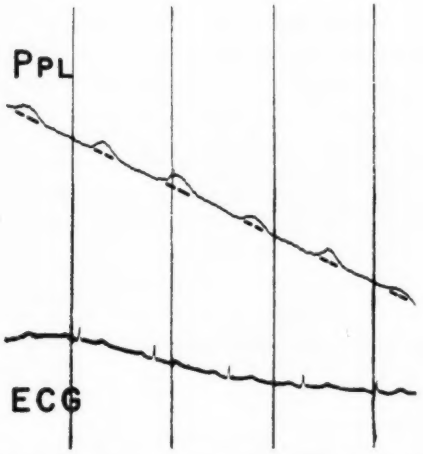

FIG. 5. PLETHYSMOGRAPHIC PRESSURE RECORD (P_{PL}) AND ELECTROCARDIOGRAM (ECG) FROM A NORMAL SUBJECT AFTER A BREATH OF NITROUS OXIDE. The slope of the pressure record follows a straight line which is periodically interrupted by upward displacements of the baseline synchronous with the heart beat. The time lines are the same as those in Figure 3. A vertical distance of 15 mm corresponds to the deflection produced in the pressure record by the introduction of 100 ml of air into the body plethysmograph.

all subjects. The patterns were not altered appreciably by the level of the inspiratory capacity at which breathholding was performed. As a rule, slight variations in amplitude occurred during successive exposures of a subject to the same test gas. Moreover, in five of the six subjects, the size of the pulsations with the different test gases varied unpredictably; only in Subject R.M. (Figure 3) were larger pulsations observed consistently with nitrous oxide than with air or helium. No correlation could be made between the density of the gas inspired and the contour of the plethysmographic pressure record.

An attempt was made to assess the effects of closing the mouth and nose (and glottis) on the pressure record. Both air and nitrous oxide were used as test gases. This maneuver was found to have no effect on the slope of the pressure curve but did diminish the amplitude of the pulsations.

Figure 7 illustrates the results obtained by the system of Figure 2. It can be seen that the rhythmic distortions in the pneumotachographic records are synchronous with those in airway pressure.

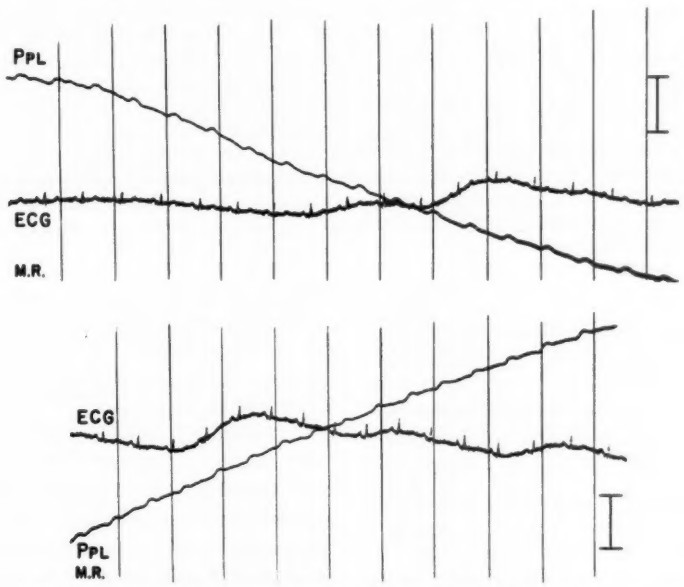

FIG. 6. PLETHYSMOGRAPHIC PRESSURE RECORDS (P_{PL}) AND ELECTROCARDIOGRAMS (ECG) FROM A NORMAL SUBJECT AFTER A BREATH OF NITROUS OXIDE. The upper half of the figure shows the usual plethysmographic record. The lower half shows a plethysmographic record the slope of which was artificially reversed to emphasize the positive nature of the deflections. The time lines and the vertical distance between brackets are the same as those in Figure 3.

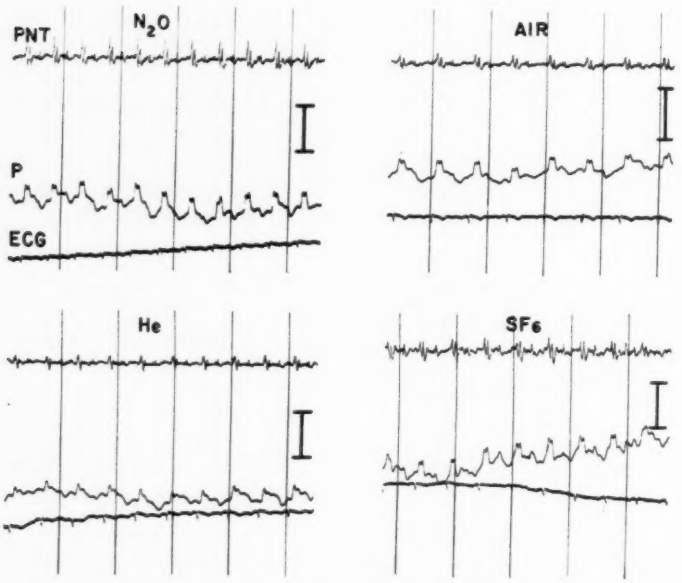

FIG. 7. PNEUMOTACHOGRAPHIC (PNT), PRESSURE (P) AND ELECTROCARDIOGRAPHIC (ECG) TRACINGS OBTAINED FROM A NORMAL SUBJECT, DURING BREATHHOLDING, USING THE SYSTEM ILLUSTRATED IN FIGURE 2. The test gases included nitrous oxide (N_2O), ambient air (air), helium (He) and sulphur hexafluoride (SF_6). Each record illustrates the interdependence of the pneumotachographic and the pressure pulsations and the electrical events of the cardiac cycle. There is a delay of 0.02 of a second between the deflections in the pneumotachographic record and the corresponding deflections in the pressure tracing. This delay arises from the different locations of the pneumotachograph and the pressure gage in the lung-bottle system (see Figure 2). Time interval between the vertical time lines is 1 second. The vertical distance between brackets equals the deflection produced in the pressure record by the introduction of 50 ml of air into the system. The upward deflections of the pneumotachographic record represent expiratory flow. The upward deflections of the pressure record represent an increase in pressure in the system.

Not illustrated is the fact that while changes in the body position of the patient do elicit changes in the pneumotachographic and pressure pulsations, they do not affect their temporal relationships to the cardiac cycle as recorded by the electrocardiogram. It should also be noted that the pressure tracings obtained from the lung-bottle system are not influenced by the same factors which determine the slope of the plethysmographic pressure records, i.e., the absorption of gas by pulmonary blood, changes in the temperature of the body plethysmograph and changes in the subject's respiratory exchange ratio. In the lung-bottle system, changes which these factors produce in the volume of gas are adjusted by automatic adaptations of the chest cage which the system is not equipped to detect.

DISCUSSION

The results indicate that the rhythmic oscillations in the plethysmographic pressure record after a single breath of nitrous oxide reflect transmission of the pulsations of the heart and great vessels rather than variations in the rate of absorption of nitrous oxide by a pulsatile pulmonary capillary blood flow.

This conclusion is supported by a consideration of the two likely mechanisms by which such oscillations could be produced:

1. If the pulsations after nitrous oxide do reflect variations in the rate of nitrous oxide uptake by pulsatile capillary flow, the records would be expected to have the following characteristics: *a*) The deflections should vary according to the absorbability of the test gas; with nitrous oxide, a negative deflection should be observed every time a blood pulse wave reached the pulmonary capillary bed; conversely, with air or nonabsorbable gases, deflections should be negligible or absent. *b*) The pattern of the pressure record obtained after a breath of nitrous oxide should differ from the pattern obtained after a breath of the other test gases; after nitrous oxide, the pressure should drop in a stepwise manner, with the fall accentuated every time a pulse of blood reached the pulmonary capillary bed; after air or the nonabsorbable gases, the pressure should follow an essentially straight line. As illustrated in Figures 3, 4, 5 and 6, none of these predictions was fulfilled.

2. On the other hand, if the pulsations arise from the impacts of the heart and great vessels, they should: *a*) occur with all test gases, and *b*) be synchronous with cardiac systole. That these conditions are fulfilled is indicated in Figures 3, 4 and 5 by the similarity in tracings obtained with the different test gases and by the relationships of the deflections to cardiac systole, i.e., their occurrence during the period of ventricular ejection (9).

Accepting the origin of the plethysmographic pressure pulsations in cardiovascular impacts, the prospect arises that variations in the amplitude of the pulsations during successive tests may result from either variation in the stroke volume of the heart or in different degrees of opening of the glottis; on the other hand, slight changes in pattern of the plethysmographic pulsations may arise from slight shifts in the position of the patient.

The data obtained in this study indicate that the pulsations in the plethysmographic record share a common origin with those in the pneumotachographic record. In both instances, the pulsations seem to arise from changes in pressure in the conducting airways produced by the mechanical events associated with cardiac systole.[1] As expected, the contour of the pulsations is somewhat different in the two types of records on at least two accounts: *1*) the plethysmograph measures changes in pressure directly, whereas the pneumotachograph measures such changes indirectly by the changes in flow which they produce; and *2*) the amplitude of the pulsations in the pneumotachographic record varies with the rate of change in pressure, whereas their amplitude in the plethysmographic record depends solely on the magnitude of the change.

It must be emphasized that the results reported in this paper do not pertain to the method proposed by Lee and DuBois for the measurement of the *mean* pulmonary capillary blood flow, i.e., the cardiac output (6). This measurement depends on the *mean* slope of the plethysmographic pressure record and is independent of any peculiarities in the contour of this record. On the other hand, their calculated values for the rate of "instantaneous" pulmonary capillary blood flow are certainly open to question since such calculations depend on the concept that the pulsations in the plethysmographic pressure record reflect a pulsatile uptake of nitrous oxide. This premise is not supported by the results of the present study.

In conclusion, the present study indicates that

[1] In spite of the convincing evidence that the cardiovascular pulsations produce rhythmic changes in pressure in the body plethysmograph, the mechanism by which such changes are produced is not completely understood. Since blood and tissues are incompressible, their simple displacement by the mechanical effects of cardiac systole would not give rise to changes in pressure in the plethysmograph. The data obtained in this study are consistent with the view that the systolic impacts affect the plethysmograph by way of the conducting airways either directly, by displacing pulses of air into and from the plethysmograph (when the mouth and glottis are open), or indirectly through rhythmic displacements of the chest wall.

Several different factors, operating singly or together, may be involved in the production of changes in pressure in the conducting airways during cardiac systole. These include: *1*) the different elastic properties of the body structures and of the air affected by the impacts, *2*) the resistance of the airways to the displacement of the air mobilized by these impacts, and *3*) the momentary imbalance between the thoracic inflow and outflow of blood during cardiac systole.

Bartlett and co-workers (8) have proposed that the momentary imbalance between thoracic inflow and outflow of blood, in conjunction with the peripheral arterial pulsations produced by the displaced blood, is responsible for the rhythmic changes in the plethysmographic pressure.

The present study does not permit distinction between the relative roles of these different factors.

the pulsations seen in the plethysmographic pressure record after a breath of nitrous oxide are due to mechanical transmission of the pulsations of the heart and great vessels and cannot be used as evidence for a pulsatile pulmonary capillary blood flow.

SUMMARY

1. The nature of the pulmonary capillary flow in man has been investigated using the body plethysmograph and various test gases.

2. Rhythmic oscillations in the plethysmographic record during breathholding with glottis open were shown to be synchronous in time with the cardiac cycle and to occur regardless of the density of the gas or its solubility in blood.

3. The rhythmic oscillations in the plethysmographic record were also shown to be synchronous with those in the pneumotachographic record.

4. Evidence is presented to indicate that both the plethysmographic and pneumotachographic records have a common origin in the mechanical events of the cardiac cycle rather than in the uptake of the test gas by pulsatile pulmonary capillary flow.

5. The conclusion is reached that the pulsations in the plethysmographic record cannot be used as evidence that pulmonary capillary flow is pulsatile.

REFERENCES

1. Hall, H. L. A study of the pulmonary circulation by the transillumination method. Amer. J. Physiol. 1925, **72**, 446.
2. Olkon, D. M., and Joannides, M. The capillary circulation in the alveolus pulmonalis of the living dog. Arch. intern. Med. 1930, **45**, 201.
3. MacGregor, R. G. Examination of the pulmonary circulation with the microscope. J. Physiol. (Lond.) 1933, **80**, 65.
4. Wearn, J. T., Ernstene, A. C., Bromer, A. W., Barr, J. S., German, W. J., and Zschiesche, L. J. The normal behavior of the pulmonary blood vessels with observations on the intermittence of the flow of blood in the arterioles and capillaries. Amer. J. Physiol. 1934, **109**, 236.
5. Ramos, J. G. On the dynamics of the lung's capillary circulation. I. The mechanical factors. Amer. Rev. Tuberc. 1955, **71**, 822.
6. Lee, G. de J., and DuBois, A. B. Pulmonary capillary blood flow in man. J. clin. Invest. 1955, **34**, 1380.
7. Lilly, J. C. Flow meter for recording respiratory flow of human subjects. Meth. med. Res. 1950, **2**, 113.
8. Bartlett, R. G., Jr., Brubach, H. F., and Specht, H. Demonstration of aventilatory mass flow during ventilation and apnea in man. J. appl. Physiol. 1959, **14**, 97.
9. Braunwald, E., Fishman, A. P., and Cournand, A. Time relationship of dynamic events in the cardiac chambers, pulmonary artery and aorta in man. Circulat. Res. 1956, **4**, 100.

THE CONCENTRATIONS OF CYSTEINE AND CYSTINE IN HUMAN BLOOD PLASMA

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Studies on the amino acid content of physiological fluids have usually been carried out under conditions that would permit any cysteine present to be oxidized to cystine prior to analysis. Consequently, little quantitative information has been obtained concerning the relative amounts of the —SH and the —S—S— forms of the amino acid in plasma. To know whether both forms are present is of particular importance in the study of excretion of amino acids by the kidney. Cystine is an amino acid which appears in abnormal quantities in the urine in several pathological conditions (1). Failure of tubular reabsorption has usually been invoked to explain this fact (2), an explanation which tacitly assumes that it is cystine (the —S—S— form) not cysteine (the —SH form) that is circulating in the blood and is cleared by the kidney. If cysteine were to be the predominant form normally circulating, oxidation to cystine in the kidney would be required in order to account for the excretion of the disulfide in the urine.

Fujita and Numata (3) and Smith and Tuller (4) have reported the presence of cysteine in blood. Another observation which could bear on the subject is that of Brand, Cahill and Harris (5) who showed that the amount of cystine in the urine of a cystinuric subject rose after feeding cysteine and methionine, but not after the ingestion of cystine, the sulfur of which appeared almost exclusively as urinary sulfate.

With these thoughts in view, a method has been developed to determine cysteine and cystine individually in the same sample of plasma. The method is based upon the fact that iodoacetate reacts rapidly with cysteine to convert it quantitatively to the stable S-carboxymethyl derivative, which can be determined chromatographically (6, 7). In the system of elution described (7, 8) for the analysis of physiological fluids with ion exchange columns, the compound emerges between urea and aspartic acid, in a position on the effluent curve not occupied by any other ninhydrin-positive substance normally found in blood plasma. Cystine is unaffected by iodoacetate and hence appears at its usual position, after valine. An effluent curve obtained by automatic recording equipment from a sample of plasma treated with iodoacetate is shown in Figure 1. In a small series of experiments, the same analytical procedure has also been found to be fairly satisfactory for the estimation of the amounts of cystine and cysteine in freshly voided urine.

MATERIALS AND METHODS

Subjects. The normal subjects (I–IV) were white healthy males ranging from the early thirties to the late forties in age. Subject V was a 19 year old Negro male who had been hospitalized for a minor stab wound of the chest about 4 days previously. The cystinuric subject (W.E.) was healthy but had suffered periodically from cystine stones which had been removed surgically on several occasions. The two subjects with Fanconi syndrome (cystine storage disease) were brothers aged 4 years (D.M.; weight 36 pounds) and 7 years (S.M.; weight 47 pounds). They presented all the findings typical of the disease [cf. Bickel, Smallwood, Smellie and Hickmans (9)], including small stature, bone changes due to rickets, cystine storage manifested by cystine crystals in the cornea, and amino aciduria. They also showed kidney damage. The younger had a blood urea nitrogen of 32 mg per 100 ml and a maximal urea clearance of 33.7 to 34.9. The corresponding values for the older brother were 79 mg per 100 ml and 7.7 to 8.7.

Samples. Somewhat more than 10 ml of blood from the antecubital vein was drawn into a dry syringe through a no. 19 gage needle. All subjects had fasted overnight. The blood was immediately ejected through the needle into a 10 ml volumetric flask that contained 100 mg of iodoacetic acid (Matheson, Coleman and Bell, East Rutherford, N. J.), 42 mg of $NaHCO_3$, and 20 mg of disodium ethylenediaminetetraacetate (EDTA), all dissolved in 1 ml of 0.1 M phosphate buffer at pH 6.8. The flask was filled to the mark with blood, and the remainder was placed in a heparinized tube for the determination

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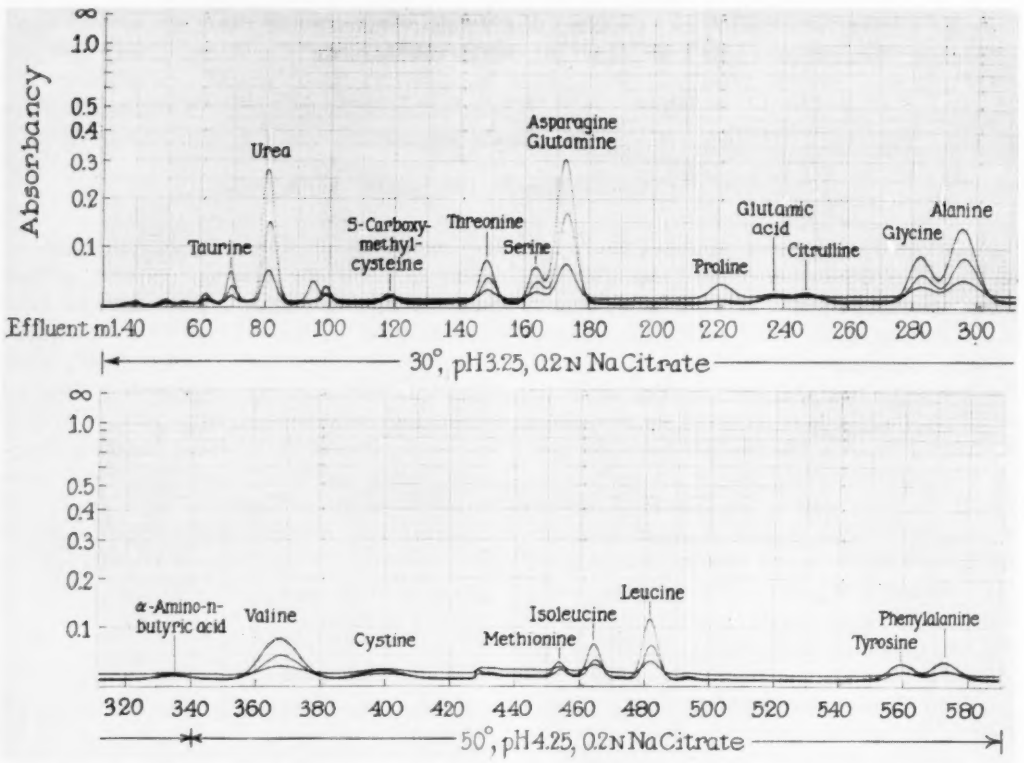

FIG. 1. CHROMATOGRAPHIC DETERMINATION OF S-CARBOXYMETHYLCYSTEINE AND CYSTINE IN IODOACETATE-TREATED NORMAL BLOOD PLASMA. The sample on the ion exchange column corresponded to 1.4 ml of normal fasting plasma. The effluent was analyzed with automatic recording equipment (7) for the acidic and neutral amino acids. The results of the analysis are tabulated in Table I (Subject 1).

of the hematocrit. If a larger or a smaller amount of blood was used, the amounts of all of the other constituents were adjusted proportionately. The plasma was separated by centrifugation (during which time the reaction of cysteine and iodoacetate proceeds to completion), deproteinized with picric acid, and freed of picric acid by the use of a bed of Dowex 1-X8, as described previously (10), except that the step wherein cysteine was oxidized to cystine was not required. The sample was concentrated almost to dryness on a rotary evaporator and washed into a 5 ml volumetric flask with buffer at pH 2.2 (7). The aliquot taken for each chromatographic analysis was 2 ml.[1] Protein-free specimens were stored, if necessary, at −20° C prior to analysis.

[1] The peaks from both S-carboxymethylcysteine and cystine are relatively small, and the steady baseline obtained with the automatic recording equipment facilitates the analysis. It would be more difficult to determine the two compounds by the manual method (8), and with either procedure the accuracy would be increased by using a larger load more closely equivalent to 4 ml of blood plasma (11).

For the analysis of urine, an aliquot of a freshly voided sample obtained from a fasting subject was added to a 10 ml volumetric flask containing the reagents given above for the treatment of blood. After 20 minutes, to allow the reaction of iodoacetate with cysteine to proceed to completion, an aliquot was removed and brought to pH 2.2 by the addition of N HCl (0.1 to 0.2 ml per ml of urine). The volume of sample chromatographed was determined on the basis of the specific gravity of the undiluted urine sample; an amount was used that corresponded to about 2 ml of urine of specific gravity 1.020 (e.g., 4 ml of urine, sp gr 1.010). Urine samples may be stored frozen under toluene after reaction with iodoacetate, although there is a loss of methionine with time.

Chromatographic analyses. All analyses were performed with the aid of the automatic recording equipment described by Spackman, Stein and Moore (7). The 30° to 50° system recommended for the analysis of physiological fluids (8) was employed [cf. Moore, Spackman and Stein (11)]. Under these conditions, S-carboxymethylcysteine emerges at about 120 ml, or 32 ml before threonine. The S-carboxymethyl derivative of reduced

glutathione, not normally observed, emerges at about 52 ml before threonine.

In calculating the amino acid content of the plasma, allowance must be made for the dilution brought about by the reagent solution present in the volumetric flask into which the freshly drawn blood is introduced. For example, if 9 ml of whole blood is added to 1 ml of reagent solution (10 ml volumetric flask), and if the hematocrit is 45 per cent (including the buffy coat), there would be 55 per cent of plasma, or $9 \times 0.55 = 4.95$ ml of the plasma before dilution. The total volume of diluted plasma is then 5.95 ml, and if 5.0 ml of diluted plasma is pipetted off after centrifugation of the cells, this volume would be the equivalent of $(4.95/5.95) \times 5.0 = 4.16$ ml of true plasma. The subsequent calculations are the same as those for the usual analysis of blood plasma (10). (The above calculation neglects the small volume correction for the solid reagents added initially.)

RESULTS

The cystine and cysteine content of plasma. A typical elution curve obtained from an iodoacetate-treated, deproteinized sample of human blood plasma is shown in Figure 1. Qualitatively, the only significant difference between this curve and one obtained from plasma not treated with iodoacetate [(11), Figure 4] is the appearance of the peak at 120 ml which is attributable to S-carboxymethylcysteine. (There also appears a small peak with maximal absorption at 440 mμ just after urea.) A cystine peak is still present, so it is clear that plasma contains both cystine and cysteine. Although iodoacetate is known to react with other functional groups of amino acids (12, 13), the alkylation of sulfhydryl groups at neutrality is by far the most rapid reaction, being complete in less than 20 minutes at pH 6 and room temperature.

The observed cystine and cysteine contents of several samples of plasma are given in the top section of Table I. The amount of the —S—S— form is between 2 and 5 times that of the —SH form. Concentrations of the other amino acids are listed in the lower part of the table; determinations of the basic amino acids were not carried out in every instance. The figures given for the neutral and acidic amino acids nearly all fall within the range of normal values determined previously (10), and the figures for cystine and cysteine thus appear to be representative for normal plasma.

TABLE I

Free amino acids of human blood plasma *

Amino acid	Normal range (10)	Subjects					Fanconi		Cystinuric
		I	II	III†	IV	V	D.M.	S.M.	W.E.
Cysteine		0.23	0.40	0.45	0.54	0.14	0.31	0.34	0.06
Cystine		0.97	0.95	0.94	1.18	0.71	1.51	1.71	0.46
Cysteine + cystine	1.08–1.30	1.20	1.35	1.39	1.74	0.85	1.82	2.05	0.52
[Per cent of cysteine + cystine as cysteine]		[19]	[30]	[32]	[31]	[16]	[17]	[20]	[11]
Taurine	0.41–0.82	0.46	0.56	0.53		0.60	1.50	0.67	0.20
Threonine	1.21–1.72	1.18	1.43	1.53		1.51	0.86	1.01	1.24
Serine	1.01–1.25	0.69	1.34	0.84		1.02	0.66	0.82	0.75
Proline	1.84–3.34	3.12	2.58	2.73		1.82	1.69	2.14	1.66
Glutamic acid	0.43–1.15	0.42	0.75	0.56		0.34	1.62	0.75	0.44
Citrulline	0.5‡	0.66	0.70	0.53		0.48	1.36	1.44	0.47
Glycine	1.34–1.73	1.12	1.58	1.34		1.41	1.28	1.55	1.10
Alanine	3.01–3.73	2.38	3.87	2.73		2.43	1.70	2.23	1.58
α-Amino-*n*-butyric acid	0.22–0.35	0.25		0.20		0.18	0.39	0.22	0.14
Valine	2.37–3.71	2.46	3.18	3.29		2.24	2.28	1.77	1.90
Methionine	0.33–0.43	0.62	0.32			0.17	0.10	0.16	0.24
Isoleucine	0.69–1.28	0.96	0.78			0.78	0.92	0.66	0.58
Leucine	1.42–2.30	1.78	1.64			1.64	1.73	1.00	1.16
Tyrosine	0.81–1.45	1.00	1.35			0.96	0.77	0.26	0.77
Phenylalanine	0.69–0.95	0.98	1.22			1.27	0.85	0.56	0.58
Ornithine	0.62–0.76					0.56		0.75	
Lysine	2.51–3.02					2.00		2.26	
1-Methylhistidine	0.04–0.17							0.03	
Histidine	0.79–1.48					0.73		0.77	
3-Methylhistidine	0.04–0.13							0.02	
Arginine	1.22–1.93					0.42		1.41	

* Subjects were all in the postabsorptive state. All values are expressed in milligrams per 100 ml of venous plasma.
† Last portion of determination was lost.
‡ Value from Archibald (14).

TABLE II

The cystine and cysteine content of urine *

Amino acid	Normal			Fanconi†		Cystinuric W.E.
	I	III	VI	D.M.‡	S.M.§	
Cysteine	4	4	3	4	4	1
Cystine	8	14	15	15	35	756
Cysteine [as per cent of cystine + cysteine]	[33]	[22]	[17]	[21]	[11]	[0.1]

* Values are expressed as milligrams per liter of fasting urine.
† The average daily urine excretion for both subjects was about 2,000 ml.
‡ The values expressed as milligrams per gram of creatinine are 39 for cysteine and 133 for cystine.
§ The values expressed as milligrams per gram of creatinine are 17 for cysteine and 131 for cystine.

In both of the children suffering from the Fanconi syndrome, the cystine in the plasma is slightly elevated, but the cysteine is in the normal range. In the cystinuric subject, both cysteine and cystine are low.

The adequacy of the iodoacetate procedure was checked by recovery experiments in which a known quantity (0.1 ml of a 1 per cent solution) of cysteine·HCl was added to one-half of a sample of whole blood prior to the iodoacetate treatment. Thereafter both halves were carried through the iodoacetate procedure and the chromatographic analyses in tandem. The recovery of added cysteine in two such experiments was 96 per cent and 98 per cent.[2]

The amount of iodoacetic acid added in the present experiments is a 50-fold excess, calculated on the basis of the total sulfhydryl content of serum (predominantly —SH of serum proteins) reported by Goldblum, Piper and Olsen (15). The results of the recovery experiments indicate that this amount of reagent is sufficient to cause complete alkylation of all of the cysteine likely to be found in plasma. Oxidation of some cysteine to cystine during the period of the reaction with iodoacetate is effectively prevented by the presence of EDTA. The chelating agent not only serves as an anticoagulant, but also protects sulfhydryl groups from the oxidation normally catalyzed by traces of metal ions. Without EDTA, cysteine in 0.003 M solution at pH 6.8 will be oxidized to the extent of 30 per cent in 45 minutes, as determined by the spectrophotometric method of Alexander (16). In the presence of the chelating agent, on the other hand, there is no decrease in sulfhydryl content during the first 60 minutes.

The cystine and cysteine content of urine. The determination of cysteine in urine as the S-carboxymethyl derivative is less precise than it is in blood. The effluent curve from urine not treated with iodoacetate (which needs to be obtained as a control) exhibits a peak very close to and partially overlapping the small carboxymethylcysteine peak, which renders the integration of the latter less certain. The recovery of cysteine added to urine was high, 113 per cent, which reflects the possible error in the integration. The conclusion that the new peak, which appears at the carboxymethylcysteine position when freshly voided urine is treated with iodoacetate, arises from cysteine is well supported by the finding that, when urine has first been allowed to stand under toluene for 24 hours at 4° C which would convert cysteine to cystine, no new peak at the carboxymethylcysteine position appears after treatment with iodoacetate.

The results of analyses for cystine and cysteine in several urine samples are given in Table II. Normal urine clearly contains both cystine and cysteine, and in about the same ratio as normal blood. The total cystine plus cysteine content is in the range previously given (17, 18) for cystine alone; the earlier values were obtained under con-

[2] The purity of the added cysteine·HCl (of known C, H, and N content) was checked by treating a portion of it with excess iodoacetate prior to chromatographic analysis. The cysteine·HCl was dissolved in 0.1 M phosphate buffer at pH 6.8 containing 0.2 per cent EDTA to stabilize the solution. Upon chromatography of an aliquot of the reaction mixture on a 15 cm column of Amberlite IR-120 at pH 3.25, the sample used was found to contain 93 per cent cysteine (recoverable as carboxymethylcysteine) and 7 per cent cystine. These values were used in calculating the recoveries from blood.

ditions which would have allowed cysteine to be oxidized to cystine prior to analysis.

The results obtained from the Fanconi patients are somewhat difficult to evaluate. The concentrations of cystine and cysteine are not markedly increased, but the amount excreted per day is abnormally large as a result of the elevation in urine volume. The ratio of cystine to creatinine is also much higher than normal. Both of the subjects excreted about 130 mg of cystine per g of creatinine, whereas the normal values for an infant lie between 28 and 54 mg, and those for a child, between 10 and 14 mg (19). Determination of the other amino acids in the urine showed these subjects to have a marked but not a massive aminoaciduria when the results are compared with those for a normal child of about the same age (20). The amino acid output was more nearly comparable with that observed for infants (19), particularly as proline and hydroxyproline were found. These two amino acids are characteristic constituents of infants' urine, but not of the urine of children and adults. The values for serine, threonine, citrulline, and lysine were particularly high.

The high cystine excretion of the cystinuric is typical for an individual suffering from this condition. The cysteine content of the urine is slightly below the normal range.

DISCUSSION

The data presented in this communication show that both cysteine and cystine are normally found in blood and urine, and that the disulfide predominates. Clearance calculations (2) for cystine in normal individuals will require reconsideration, but the changes in the numerical values will be small since the ratio of the two forms in urine is found to be similar to that in the blood. Cystinuria is clearly associated with a high rate of clearance of cystine (2); there is as yet no evidence that blood cysteine is the precursor of cystine in the urine in this abnormality, even though Brand, Cahill and Harris (5) have shown that ingestion of cysteine (but not cystine) led to increased excretion of cystine in the urine. It is apparent that the metabolic relationships between the —SH and —S—S— forms of the amino acid are not fully understood. Methods such as the present one may permit further studies of the relationships of the two forms in different tissues as well as additional investigations of their excretion by the kidney in normal and pathological conditions.

SUMMARY

1. A method has been developed for the determination of cystine and cysteine in the same sample of fresh blood or urine. Cysteine is determined by converting it immediately to the stable S-carboxymethyl derivative by reaction with iodoacetate. The other amino acids are not affected. The amino acid composition, including the S-carboxymethylcysteine content, is determined by ion exchange chromatography on columns of sulfonated polystyrene resins.

2. Normal human plasma obtained from individuals in the postabsorptive state contains about 1 mg of cystine and 0.4 mg of cysteine per 100 ml. The values for a single cystinuric subject were both slightly low, whereas the cystine (but not the cysteine) content of the plasma of two subjects suffering from the Fanconi syndrome was somewhat high.

3. The normal individual excretes, in the fasting state, 10 to 15 mg of cystine and 2 to 4 mg of cysteine per L of urine. The two Fanconi subjects, both of whom exhibited aminoaciduria, had an elevated cystine excretion, but the cysteine content of the urine was essentially normal. In a cystinuric, the cystine excretion was massive, but the cysteine concentration was slightly subnormal.

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REFERENCES

1. Dent, C. E., and Walshe, J. M. Amino-acid metabolism. Brit. med. Bull. 1954, **10**, 247.
2. Arrow, V. K., and Westall, R. G. Amino acid clearances in cystinuria. J. Physiol. 1958, **142**, 141.
3. Fujita, A., and Numata, I. Über die kolorimetrische Bestimmung von Cystein und Cystin. Biochem. Z. 1938–39, **300**, 264.
4. Smith, E. L., and Tuller, E. F. The paper chromatographic detection of the free-sulfur containing

amino acids and small peptides in whole blood and serum. Arch. Biochem. 1955, **54**, 114.

5. Brand, E., Cahill, G. F., and Harris, M. M. Cystinuria II. The metabolism of cystine, cysteine, methionine, and glutathione. J. biol. Chem. 1935, **109**, 69.
6. Moore, S., Cole, R. D., Gundlach, H. G., and Stein, W. H. On the cleavage of disulfide bonds by reduction *in* Proceedings of IVth Internat. Congr. of Biochemistry, Vienna, 1958; Symposium VIII, Proteins. New York, Pergamon Press, 1958, p. 52.
7. Spackman, D. H., Stein, W. H., and Moore, S. Automatic recording apparatus for use in the chromatography of amino acids. Analyt. Chem. 1958, **30**, 1190.
8. Moore, S., Spackman, D. H., and Stein, W. H. Chromatography of amino acids on sulfonated polystyrene resins. An improved system. Analyt. Chem. 1958, **30**, 1185.
9. Bickel, H., Smallwood, W. C., Smellie, J. M., and Hickmans, E. M. Clinical description, factual analysis, prognosis and treatment of Lignac-Fanconi disease. Acta paediat. Uppsala 1952, **42**, Suppl. 90, p. 27.
10. Stein, W. H., and Moore, S. Free amino acids of human blood plasma. J. biol. Chem. 1954, **211**, 915.
11. Moore, S., Spackman, D. H., and Stein, W. H. Automatic recording apparatus for use in the chromatography of amino acids. Fed. Proc. 1958, **17**, 1107.
12. Korman, S., and Clarke, H. T. Carboxymethylamino acids and peptides. J. biol. Chem. 1956, **221**, 113.
13. Gundlach, H. G., Stein, W. H., and Moore, S. The nature of the amino acid residues involved in the inactivation of ribonuclease by iodoacetate. J. biol. Chem. 1959, **234**, 1754.
14. Archibald, R. M. Determination of citrulline and allantoin and demonstration of citrulline in blood plasma. J. biol. Chem. 1944, **156**, 121.
15. Goldblum, R. W., Piper, W. N., and Olsen, C. J. Studies on sulfhydryl groups in sera of normal humans. J. invest. Derm. 1954, **22**, 373.
16. Alexander, N. M. Spectrophotometric assay for sulfhydryl groups using N-ethylmaleimide. Analyt. Chem. 1958, **30**, 1292.
17. Stein, W. H. Chromatographic investigation of the amino acid constituents of normal urine. J. biol. Chem. 1953, **201**, 45.
18. Evered, D. F. Excretion of amino acids by the human; a quantitative study with ion-exchange chromatography. Biochem. J. 1956, **62**, 416.
19. Fowler, D. I., Norton, P. M., Cheung, M. W., and Pratt, E. L. Observations on the urinary amino acid excretion in man: The influence of age and diet. Arch. Biochem. 1957, **68**, 452.
20. Jonxis, J. H. P., and Huisman, T. H. J. Amino aciduria and ascorbic acid deficiency. Pediatrics 1954, **14**, 238.

THE LIPID COMPOSITION OF SERUM IN PATIENTS WITH LIVER DISEASE *

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The association of a serum lipid abnormality and liver disease has been known since 1862, when Flint (1) described the accumulation of "cholesterine" in the blood of patients with liver disease as the result of decreased excretion of this substance into the bile. Subsequent observations disclosed that this increase in serum cholesterol concentration was largely in the unesterified (free) fraction, so that the ratio of free/total cholesterol was elevated (2–4). Man, Kartin, Durlacher and Peters (5), in 1945, showed that the lipid phosphorus concentration and the ratio of lipid phosphorus/cholesterol were also elevated in certain patients with liver disease. The elevation in serum lipid phosphorus concentration appeared to be attributable largely to an increase in the serum lecithin concentration (6–9). The hyperlipemia and the increase in the ratio of free/total cholesterol, moreover, showed some correlation with the degree of jaundice, whether due to biliary obstruction or to parenchymal damage (5, 10–12).

Abnormalities in the serum lipoprotein composition have also been described in patients with liver disease and jaundice. Marked increases in the S_f6 and S_f8 with varying increases in the S_f10–17 ultracentrifugal classes of lipoproteins have been reported in patients with biliary cirrhosis (13). In patients with hepatitis, increases in the S_f0–12, 12–20, and 20–100 classes, which correlated positively with the icteric index, and decreases in the S_f100–400 class, which correlated negatively with the icteric index, have been found (14). Increased concentrations of β-lipoprotein and decreased-to-absent concentrations of α-lipoprotein have been observed with zone electrophoresis of serum from patients with jaundice (15). Similar findings have been reported with filter paper electrophoresis (16, 17) and ultracentrifugation (17–19). Abnormalities in these lipoprotein fractions, moreover, have been demonstrated by both chemical (15, 17, 19) and immunological means (20). By ultracentrifugal analysis, Havel, Eder and Bragdon (19) demonstrated a decreased ratio of cholesterol/phospholipid in the fractions with density (D) < 1.019, 1.019–1.063, and > 1.063 as well as an increase in the concentration of the fraction D < 1.063 and a decrease in the concentration of the fraction D > 1.063.

In the present investigation, a chromatographic technique for the estimation of the main individual phospholipids of serum (21, 22) was employed in order to characterize further the serum lipid abnormalities of patients with liver disease. The lipids were analyzed in this way in the whole serum of 12 patients with parenchymatous or obstructive jaundice, 3 patients with cirrhosis without jaundice, and 3 patients with bile fistula. In 10 of these patients, in addition, the lipids of the ultracentrifugally separated serum fractions D < 1.019, < 1.063, and > 1.063 were analyzed in the same manner.

MATERIALS AND METHODS

Six male and 12 female patients on the wards of the Presbyterian Hospital were studied. A brief description follows.

M.N., D.C., N.W. and P.K. were females with jaundice of 8 to 16 years' duration, and hepatosplenomegaly. Laboratory studies of the serum showed negative cephalin flocculation reactions and elevated alkaline phosphatase concentrations in all and a low albumin concentration in 3 of the 4 patients tested; these were classified as having primary biliary cirrhosis. Patient M.N., in addition, had diabetes mellitus known for 3 years. At the time of the serum lipid analysis, M.N. was receiving bile salts orally and P.K. had been on prednisone, 10 mg per day for 1 week.

Patient L.U. had jaundice of 8 years' duration secondary to postoperative bile duct stricture. Patient K.M.

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had jaundice of the obstructive type with a negative cephalin flocculation reaction and an elevated serum alkaline phosphatase concentration. The jaundice, which started about 11 days before the serum lipid analysis, rapidly decreased during the week before the serum lipid analysis, and it was felt that this spontaneously reversible jaundice was due either to passage of a common duct stone, as stabbing right upper quadrant pain was present, or to the ingestion of chlorpromazine, which was stopped about 10 days prior to the onset of jaundice.

Patient I.H. was in hepatic coma, at the time of the serum lipid analysis, as a result of the acute hepatic insufficiency of the chronic alcoholic (23) and died two days later. His liver and spleen were not palpable and the cephalin flocculation reaction was 3+. He had received a total of 40 mg of prednisone during the 2 days prior to the serum lipid analysis.

Patients J.E., G.L., M.C., E.E. and J.M. had had typical acute viral hepatitis with jaundice for 1 to 4 weeks before the serum lipid analysis. All had hepatomegaly except J.E., in whom the liver was not felt, and all had 3+ or 4+ cephalin flocculation reactions except J.E., who had a negative cephalin flocculation reaction but a 2+ thymol turbidity reaction. Only E.E. had a low serum albumin concentration, which was about 40 per cent of the normal.

Patients M.M., E.H. and J.C. had cirrhosis apparently on the basis of chronic alcoholism. All had firm enlarged livers and M.M. and E.H. had ascites and edema. The cephalin flocculation reaction was 2+ in M.M., 3+ in E.H., and negative in J.C. J.C. had 20 per cent retention of sulfobromophthalein at 30 minutes after administration.

Patients H.S. and F.M. were draining bile through common bile duct T-tubes following common bile duct exploration for choledocholithiasis. At the time of the lipid analysis, H.S. had drained about 275 ml of bile per day for 10 days and F.M. about 450 ml of bile per day for 8 days. L.K. had had a cholecystostomy which was performed for acute cholecystitis; because of a postoperative myocardial infarction, further surgery was delayed. At the time of the lipid analysis, which was done about 6 months after the cholecystostomy was performed, L.K. was draining about 150 ml of bile per day.

The direct serum bilirubin concentrations were measured in the hospital routine laboratory by the method of Malloy and Evelyn (24) and are reported in Table I. The values reported were obtained within 1 to 2 days of the serum lipid analyses except in Patients M.N. (11 days before), D.C. (5 days before), E.H. (8 days before) and L.K. (4 days before).

All of the serum samples were obtained from fasting patients. A lipid extract of each serum sample was prepared within 6 hours after withdrawal and stored *in vacuo* at $-30°$ C for as long as several weeks. The lipid extract was then chromatographed in duplicate on silicic acid, using chloroform-methanol-water mixtures for elution, in order to separate and quantitate the individual phospholipids, cholesterol, cholesterol ester, and triglyceride. In 10 patients, aliquots of the serum samples were also adjusted as to density and ultracentrifuged in order to separate the fractions D < 1.019, < 1.063, and > 1.063. In L.U. the 3 fractions separated were D < 1.019, 1.019–1.063, and > 1.063. All of the ultracentrifugally separated fractions were then extracted and chromatographed in the same manner as the whole serum samples. The methods used for extraction, chromatography and ultracentrifugation have been described previously (21, 22, 25). The column fractions were analyzed for phosphorus by the method of Fiske and Subbarow (26), for cholesterol and cholesterol ester by the method of Schoenheimer and Sperry (27) as modified by Sperry and Webb (28), and for ester bonds by the method of Rapport and Alonzo (29). Serum triglyceride concentration was calculated by subtracting the serum cholesterol ester concentration from the total serum nonphosphorus-containing lipid ester concentration and dividing by 3.

The normal values used in Table I for whole serum lipids include a combined series of 7 previously reported (22) and of 8 previously not reported; the values for the ultracentrifugally separated serum fractions of this latter group, however, have been reported (25) except for cholesterol, cholesterol ester and triglyceride, and are the normal values quoted in Tables II to VII. These 15 normal values were obtained from 10 male and 5 female subjects with an average age of 32 ± 11 (SD) years.

The estimation of lysolecithin by the column chromatographic method used is not precise, one reason being that lysolecithin comprises only about 7 to 8 per cent of the total phospholipid of normal human serum; a more important reason, however, is that the lysolecithin fraction, which is the final phosphorus-containing fraction eluted from the column, is contaminated with lecithin and sphingomyelin. In a previous communication (21), it was shown that this final fraction obtained on chromatography of normal whole serum lipid extract was composed predominantly of lysolecithin, but contained about 10 per cent lecithin and 10 per cent sphingomyelin. In calculating this final fraction, therefore, 80 per cent was considered to be lysolecithin, and 10 per cent was added to the lecithin and 10 per cent to the sphingomyelin fraction (22). On chromatographing samples of lecithin and sphingomyelin containing no lysolecithin, however, these substances were found in the final phosphorus-containing fraction, and it appeared that the amount of lecithin and sphingomyelin in this final fraction was related to the total amount of material in the lecithin and sphingomyelin fractions, respectively, of the lipid extract; thus, by subtracting from this final fraction the appropriate percentage of the lecithin and sphingomyelin fractions, a more accurate approximation of the lysolecithin concentration should be obtained. To determine the respective percentages of the lecithin and sphingomyelin fractions appearing in the final fraction, 5 lipid extracts of normal human serum were chromatographed in amounts ranging from 10 to 18 μmoles of lipid phosphorus per g of silicic acid. When the final fraction of each was analyzed by

rechromatography, an average of 1.3 (range 0.9 to 1.6) per cent of the lecithin fraction and 4.9 (range 2.3 to 7.9) per cent of the sphingomyelin fraction appeared in the final fraction, these values showing no correlation with the amount of lipid phosphorus chromatographed per gram of silicic acid. Using these figures, therefore, all of the normal values recorded here were recalculated from the reported data so that 1.3 per cent of the lecithin fraction and 4.9 per cent of the sphingomyelin fraction were considered to be in the final fraction. Thus, these amounts were subtracted from the lysolecithin fraction and added, respectively, to the lecithin and sphingomyelin fractions, the rest of the final fraction being considered as lysolecithin. The reported values for the fraction with density > 1.063 (*25*) had already been calculated in this way. The values for the patients in the present series were calculated in the same way. When 3 samples of normal serum lecithin were chromatographed in amounts of 3.5, 7.5 and 11.3 μmoles lipid phosphorus per g of silicic acid, 1.8, 1.2 and 1.4 per cent of the total lecithin, respectively, appeared in the final fraction. Two samples of whole human red blood cell lipid extract, which contained about one-half the relative lecithin concentration as human serum, when chromatographed in the same way, showed 1.0 to 1.1 per cent of the lecithin fraction in the final fraction. The markedly decreased concentrations of lysolecithin reported in the present study, which were reflected by a decrease in the concentrations of the uncorrected lysolecithin fraction, therefore, do not appear to be related to the associated high concentration of lecithin found. Confirmatory evidence for the decreased lysolecithin concentration was obtained, moreover, by estimating the size of the spots obtained by chromatography of the lipid extract on silicic acid-impregnated paper.

The concentrations of the individual phospholipids in the density fractions were calculated on the basis of the actual amounts of phosphorus eluted from the column. The corresponding normal values reported previously (*25*) were calculated on the basis of 100 per cent recovery from the column. Since the average recovery was less than 100 per cent, these normal values were recalculated on the basis of the actual amounts of phosphorus eluted from the column, and appear in Tables III–V.

RESULTS

Whole serum

The values for the concentrations of the various lipids measured in the whole serum of all of the patients studied are presented in Table I. These patients can be divided into three groups: *1*) patients with jaundice (the top 12 in Table I) with direct serum bilirubin concentration greater than 1.8 mg per 100 ml, *2*) patients with cirrhosis with direct serum bilirubin concentration less than 1.8 mg per 100 ml, and *3*) patients with bile fistula.

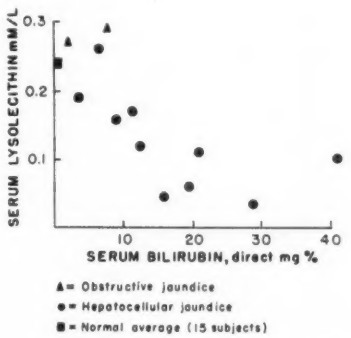

FIG. 1. THE RELATIONSHIP OF SERUM LYSOLECITHIN CONCENTRATION TO DIRECT SERUM BILIRUBIN CONCENTRATION IN PATIENTS WITH JAUNDICE.

Patients with jaundice. In these 12 patients, the total lipid phosphorus concentration varied over a wide range. The lecithin concentration was elevated [1] in 8 of the 12 patients and correlated well with the total lipid phosphorus concentration, as it comprised about three-fourths of the latter. The sphingomyelin and "cephalin" concentrations appeared to correlate, but less well, with the total lipid phosphorus concentration. The sphingomyelin concentration tended to be normal but was elevated in the three patients with the highest total lipid phosphorus concentrations, and the "cephalin" concentration was elevated. The lysolecithin concentration was lowered in all patients with a direct serum bilirubin concentration greater than 11.4 mg per 100 ml, and an inverse correlation between the concentrations of serum lysolecithin and direct serum bilirubin is suggested in Figure 1. The two patients with the obstructive type of jaundice, whose direct serum bilirubin concentrations were 7.6 and 1.8 mg per 100 ml, respectively, had normal lysolecithin concentrations. The lowest lysolecithin concentration was seen in the patient (I.H.) with acute hepatic insufficiency and coma.

The relative amounts of lecithin and sphingomyelin showed more consistent changes than did the absolute amounts. The percentage of the total phospholipid analyzed as lecithin was elevated in 11 and the sphingomyelin/lecithin ratio was decreased in all of the patients (Figure *2*). The

[1] A value designated as elevated or lowered means that it is beyond twice the standard deviation from the average.

TABLE 1

The serum lipid composition of patients with liver disease

Patient	Diagnosis	Sex	Age	Serum bilirubin direct	Phospholipids*								Recovery	Serum lipid P	Cholesterol			Non-P serum lipid ester	Triglyceride
					"Cephalin"		Lecithin		Sphingomyelin		Lysolecithin				Free	Total	Free		
				mg./100ml	%	*mmoles/L*	%	*mmoles/L*	%	*mmoles/L*	%	*mmoles/L*	%	*mmoles/L*	*mmoles/L*	*mmoles/L*	%	*mmoles/L*	*mmoles/L*
Normals [15]	Mean				5.0	0.15	68.2	2.10	19.0	0.59	7.7	0.24	96	3.22	1.21	4.62	26.2	5.64	0.74
	SD				0.6	0.03	1.7	0.31	1.9	0.12	1.5	0.05	3	0.49	0.20	0.71	1.6	0.87	0.29
M.N.	Primary biliary cirrhosis	F	52	12.3	4.8	0.21	80.7	3.59	11.9	0.53	2.6	0.12	95	4.68	3.52	6.75	52.1	7.03	1.27
D.C.	Primary biliary cirrhosis	F	39	40.8	3.9	0.14	79.9	2.96	13.4	0.50	2.8	0.10	93	3.98	3.05	3.23	94.4	6.67	2.16
N.W.	Primary biliary cirrhosis	F	57	11.4	4.4	0.44	78.3	7.90	15.6	1.58	1.7	0.17	100	10.10	12.10	13.10	92.4	5.94	1.65
P.K.	Primary biliary cirrhosis	F	50	19.6	6.0	0.18	75.9	2.27	16.1	0.48	2.0	0.06	96	3.12	3.15	4.11	76.6	7.24	2.09
L.U.	Obstructive jaundice	F	46	7.6	4.3	0.31	75.4	5.51	16.3	1.19	4.0	0.29	90	8.13	4.42	8.69	51.0	8.15	1.29
K.M.	Obstructive jaundice	F	60	1.8	6.0	0.32	72.6	3.87	16.4	0.87	5.0	0.27	99	5.38	3.00	8.66	34.6	15.10	3.15
I.H.	Acute hepatic insufficiency	M	71	28.8	3.8	0.10	77.7	2.05	17.2	0.45	1.3	0.03	96	2.75	2.30	2.77	83.0	6.24	1.92
J.E.	Hepatitis	M	70	6.2	6.0	0.22	73.9	2.70	13.1	0.48	7.0	0.26	90	4.06	2.16	6.04	35.8	12.24	2.79
G.L.	Hepatitis	F	55	20.8	8.1	0.31	73.0	2.82	16.0	0.62	2.8	0.11	101	3.82	3.15	4.16	75.7	11.34	3.44
M.C.	Hepatitis	F	33	9.2	7.8	0.31	73.5	2.96	14.8	0.60	3.9	0.16	102	3.94	2.82	4.45	63.4	8.25	2.21
E.E.	Hepatitis	F	28	16.0	6.6	0.27	76.5	3.16	15.8	0.65	1.1	0.05	100	4.14	3.80	4.06	93.6	8.18	2.64
J.M.	Hepatitis	M	17	3.6	5.6	0.15	70.2	1.82	16.9	0.44	7.3	0.19	103	2.52	1.58	4.99	31.7	7.25	1.28
M.M.	Cirrhosis	F	68	0.8	5.3	0.15	69.5	1.91	20.3	0.56	4.9	0.13	105	2.62	1.51	4.68	32.2	5.75	0.86
E.H.	Cirrhosis	M	66	1.2	5.7	0.13	72.0	1.61	17.7	0.40	4.6	0.10	88	2.54	1.03	2.98	34.6	4.44	0.83
J.C.	Cirrhosis	M	51	0.9	4.4	0.14	70.9	2.20	17.4	0.54	7.4	0.23	82	3.78	1.54	5.33	28.9	7.46	1.22
H.S.†	Bile fistula	F	38		5.2	0.19	70.7	2.62	17.0	0.63	7.1	0.26		3.71	1.25	4.55	27.5	9.82	2.17
L.K.	Bile fistula	F	70	0.6	5.2	0.16	68.9	2.16	19.9	0.62	6.1	0.19	97	3.23	1.48	4.40	33.7	8.44	1.84
F.M.	Bile fistula	M	55	2.0	5.6	0.16	67.5	1.87	18.9	0.52	8.0	0.22	94	2.95	1.34	3.70	36.4	7.39	1.68

* Phospholipid values are expressed as millimoles of phosphorus.
† Calculated on basis of D < 1.063 and > 1.063 fractions.

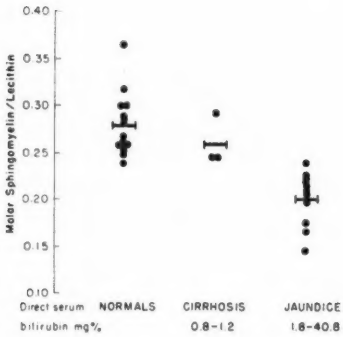

FIG. 2. THE MOLAR SPHINGOMYELIN/LECITHIN RATIOS OF SERUM.

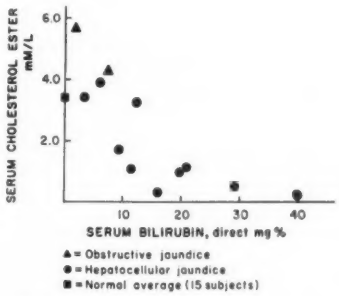

FIG. 3. THE RELATIONSHIP OF SERUM CHOLESTEROL ESTER CONCENTRATION TO DIRECT SERUM BILIRUBIN CONCENTRATION IN PATIENTS WITH JAUNDICE.

percentage of the total phospholipid analyzed as "cephalin" tended to be elevated in the patients with hepatitis.

The free cholesterol concentration as well as the ratio of free/total cholesterol was elevated in all of these patients but one, while the total cholesterol concentration varied considerably. The cholesterol ester concentration, like the lysolecithin concentration, appeared to be inversely related to the direct serum bilirubin concentration, as is seen in Figure 3.

The serum triglyceride concentration was elevated in nine of the patients. All of the sera were clear on inspection except that of Patient J.M., whose serum showed a faint opalescence.

Patients with cirrhosis. The three patients with cirrhosis did not show the lipid changes seen in the patients with jaundice, except for some elevation of the ratio of free/total cholesterol in two, lowering of the cholesterol ester concentration in one, and lowering of the concentration of lysolecithin in two. The latter two patients had ascites and edema. All of the sera were clear on inspection.

Patients with bile fistula. The three patients with bile fistula likewise did not show the changes seen in the patients with jaundice except for an

TABLE II

Distribution of serum lipid phosphorus in density fractions

Patient	Diagnosis	D<1.019		D<1.063		D>1.063		Recovery*	Total serum lipid P
		mmoles/L	*% total*	*mmoles/L*	*% total*	*mmoles/L*	*% total*	*%*	*mmoles/L*
Normals	Average	0.49	18.0	1.37	45	1.65	55	94	3.21
[8]	SD	0.29	10.6	0.35	6	0.27	6	5	0.62
N.W.	Primary biliary cirrhosis	0.28	2.8	9.70	96	0.42	4	100	10.1
L.U.	Obstructive jaundice	0.67	10.0	5.18†	77†	1.55	23	87	7.72
K.M.	Obstructive jaundice	0.98	20.1	3.90	80	0.97	20	91	5.38
I.H.	Acute hepatic insufficiency	‡	‡	2.22	90	0.24	10	90	2.75
J.E.	Hepatitis	6.67	15.9	2.98	71	1.23	29	104	4.06
M.M.	Cirrhosis	0.49	18.5	1.11	42	1.51	58	100	2.62
E.H.	Cirrhosis	0.54	21.6	1.23	49	1.27	51	98	2.54
J.C.	Cirrhosis	0.30	9.3	1.67	53	1.50	47	84	3.78
H.S.	Bile fistula	1.10	30.7	2.04	57	1.54	43	96	3.71
L.K.	Bile fistula	0.64	18.9	1.82	54	1.57	46	105	3.23

* Per cent recovery of total serum lipid P from 1.063 density tube after centrifugation.
† Calculated by adding D < 1.019 and D1.019–1.063 fractions.
‡ Sample lost.

TABLE III

Phospholipid composition of fraction with density < 1.019 *

Patient†	Diagnosis	"Cephalin"		Lecithin		Sphingomyelin		Lysolecithin	
		mmoles/L	*% total*	*mmoles/L*	*% total*	*mmoles/L*	*% total*	*mmoles/L*	*% total*
Normals [4]	Average	0.032	5.7	0.39	66.4	0.15	23.5	0.028	4.5
L.U.	Obstructive jaundice	0.034	5.4	0.46	73.0	0.12	18.7	0.018	2.9
K.M.	Obstructive jaundice	0.061	6.5	0.67	72.0	0.18	19.0	0.023	2.5
J.E.	Hepatitis	0.049	6.8	0.51	70.4	0.14	19.0	0.028	3.9
L.K.	Bile fistula	0.045	8.0	0.36	64.5	0.13	24.0	0.019	3.5

* Phospholipid values are expressed as millimoles of phosphorus.
† Samples on other patients either not done or too inaccurate because of small quantity.

TABLE IV

Phospholipid composition of fraction with density < 1.063 *

Patient	Diagnosis	"Cephalin"		Lecithin		Sphingomyelin		Lysolecithin	
		mmoles/L	*% total*	*mmoles/L*	*% total*	*mmoles/L*	*% total*	*mmoles/L*	*% total*
Normals [8]	Average	0.053	4.4	0.87	66.6	0.34	26.0	0.039	3.0
	SD	0.008	0.6	0.21	1.1	0.08	1.5	0.014	0.8
N.W.	Primary biliary cirrhosis	0.427	4.4	7.60	78.3	1.51	15.6	0.165	1.7
L.U.†	Obstructive jaundice	0.198	3.9	3.85	75.9	0.88	17.4	0.142	2.8
K.M.	Obstructive jaundice	0.191	5.1	2.86	76.5	0.61	16.4	0.071	1.9
I.H.	Acute hepatic insufficiency	0.072	3.5	1.64	79.6	0.33	16.0	0.017	0.8
J.E.	Hepatitis	0.145	5.4	2.02	74.8	0.48	17.7	0.060	2.2
M.M.	Cirrhosis	0.048	4.6	0.74	70.6	0.24	22.3	0.027	2.6
E.H.	Cirrhosis	0.060	5.5	0.77	71.4	0.23	21.4	0.019	1.8
J.C.	Cirrhosis	0.063	4.0	1.10	69.8	0.38	23.8	0.038	2.4
H.S.	Bile fistula	0.102	5.1	1.39	69.6	0.44	21.9	0.066	3.3
L.K.	Bile fistula	0.097‡	5.6‡	1.19	68.7	0.41	23.6	0.037	2.2

* Phospholipid values are expressed as millimoles of phosphorus.
† Calculated by adding D < 1.019 and D1.019–1.063 fractions.
‡ Estimated.

TABLE V

Phospholipid composition of fraction with density > 1.063 *

Patient†	Diagnosis	"Cephalin"		Lecithin		Sphingomyelin		Lysolecithin	
		mmoles/L	*% total*	*mmoles/L*	*% total*	*mmoles/L*	*% total*	*mmoles/L*	*% total*
Normals [8]	Average	0.087	5.2	1.15	70.0	0.22	13.6	0.18	11.1
	SD	0.025	0.7	0.22	2.1	0.05	1.3	0.03	2.8
L.U.	Obstructive jaundice	0.064	4.0	1.27	78.6	0.13	8.3	0.146	9.0
K.M.	Obstructive jaundice	0.065	6.2	0.78	73.9	0.11	10.5	0.099	9.4
J.E.	Hepatitis	0.073	6.9	0.69	65.1	0.11	10.5	0.185	17.5
M.M.	Cirrhosis	0.079	5.5	1.02	71.2	0.24	16.6	0.098	6.8
E.H.	Cirrhosis	0.076	6.6	0.82	71.7	0.18	15.8	0.068	6.0
J.C.	Cirrhosis	0.065	5.3	0.86	70.5	0.15	11.9	0.150	12.3
H.S.	Bile fistula	0.082	5.3	1.11	72.1	0.16	10.4	0.188	12.2
L.K.	Bile fistula	0.082	5.5	1.07	71.8	0.22	15.0	0.115	7.7

* Phospholipid values are expressed as millimoles of phosphorus.
† The values for N.W. and I.H. not recorded because of inaccuracy due to small quantity of fraction.

elevation of the ratio of free/total cholesterol in two and of the triglyceride concentration in all. The lysolecithin concentration was within the normal range. The sera of Patients H.S. and L.K. showed a faint opalescence, while the serum of F.M. was clear on inspection.

Serum lipoprotein fractions. In ten patients, aliquots of the same samples used for whole serum analysis were adjusted as to density, and fractions D < 1.019, < 1.063 and > 1.063 were separated by ultracentrifugation. The values for the lipid phosphorus in each density fraction are given in Table II. A striking elevation of the relative amount of lipid phosphorus in the fraction D < 1.063 is evident in the five patients with jaundice, even when the total serum lipid phosphorus was not elevated (I.H.). The absolute amount of lipid phosphorus was elevated in the fraction D < 1.063 in all of the five and was lowered in the fraction D > 1.063 in three of the five. These changes were in contrast to the minimal, if any, deviations from normal observed in the patients with cirrhosis and with bile fistula. No consistent changes in the relative or absolute amount of total lipid phosphorus in the fraction D < 1.019 were seen. The fraction D > 1.21 was analyzed in Patients L.U. and L.K. and found to contain about 2 per cent and 4 per cent, respectively, of the total lipid phosphorus.

The values for the individual phospholipids in each density fraction are given in Tables III to V. The changes in the relative concentrations of the individual phospholipids as compared with the normal values were in the same direction as in whole serum. The sphingomyelin/lecithin ratio in the fraction D < 1.063 was considerably lower in the patients with jaundice than in those with cirrhosis and with bile fistula. This ratio, however, was considerably higher in the fraction D < 1.063 than in the fraction D > 1.063, as in normal subjects (25), suggesting that the relative binding affinities for these phospholipids by the respective lipoproteins were similar to the normal. The molar "cephalin"/sphingomyelin ratio was higher in the fraction D > 1.063 than in the fraction D < 1.063, as in normal subjects (25), suggesting that the relative binding affinities for these phospholipids by the respective lipoproteins were also similar to the normal. The "cephalin"/

TABLE VI

Distribution of serum cholesterol in density fractions

Patient	D<1.019				D<1.063				D>1.063				Recovery*		Total serum cholesterol	
	Free	Total	FC† TC	Free	Free	Total	FC TC	Free	Free	Total	FC TC	Total	Free	Total	Free	Total
	mmoles/L.		%	*% total‡*	*mmoles/L.*		%	*% total*	*mmoles/L.*		%	*% total*	%		*mmoles/L.*	
Normals [6]	0.27	0.91	31	32	26	0.84	2.91	29	77	68	0.25	1.37	19	23	32	89	89	1.22	4.79
Average§						11.39	12.60	90	94	96						94	96	12.10	13.10
N.W.	0.32	0.85	38	9	11	3.32‖	6.61‖	50	90‖	77‖	0.36	0.87	41	10	12	83	86	4.42	8.69
L.U.	0.64	1.44	44	23	18	2.60	7.06	37	94	88	0.16	0.97	17	6	12	92	93	3.00	8.66
F.M.						1.99	2.33	85	93	92	0.16	0.20	80	7	8	93	91	2.30	2.77
I.H.	0.49	1.33	37	25	23	1.79	5.25	34	91	91	0.18	0.52	35	9	9	91	96	2.16	6.04
M.M.	0.26	0.75	35	19	19	0.75	2.20	33	56	55	0.60	1.81	33	44	45	90	86	1.51	4.68
E.H.	0.23	0.80	29	23	28	0.72	1.88	38	72	65	0.28	1.01	28	28	35	97	97	1.03	2.98
J.C.	0.23	0.45	51	17	9	1.15	3.82	35	85	81	0.20	0.91	22	15	19	87	89	1.54	5.33
H.S.						1.03	3.41	30	82	75	0.22	1.14	19	18	25			1.25‖	4.55‖
L.K.	0.28	0.75	37	21	17	1.18	3.21	37	81	76	0.25	1.02	25	19	24	97	96	1.48	4.40

* Per cent recovery of total serum cholesterol from 1.063 density tube after centrifugation.
† Free cholesterol/total cholesterol × 100.
‡ Free or total cholesterol of fraction/free or total cholesterol of D <1.063 + >1.063 fractions, respectively, × 100.
§ Calculated by adding D <1.019 and 1.109–1.063 fractions.
‖ Calculated by adding D <1.063 and >1.063 fractions.

TABLE VII

Distribution of serum triglyceride in density fractions

Patient	D <1.019		D <1.063		D> 1.063		Recovery*	Total serum triglyceride
	mmoles/L	*% total*	*mmoles/L*	*% total*	*mmoles/L*	*% total*	*%*	*mmoles/L*
Normals [5] Average			0.54	85	0.10	15		0.64†
N.W.			1.55	94				1.65
K.M.	1.85	73	2.38	94	0.15	6	80	3.15
I.H.			1.62	95	0.08	5	88	1.92
J.E.	1.57	55	2.62	92	0.24	8	102	2.79
M.M.	0.47	55	0.52	61	0.33	39	100	0.86
E.H.	0.33	42	0.60	78	0.17	22	93	0.83
J.C.	0.60	48	1.05	85	0.19	15	101	1.22
H.S.			1.92	88	0.25	12		2.17†
L.K.	1.11	67	1.42	86	0.23	14	90	1.84

* Per cent recovery of total serum triglyceride from 1.063 density tube after centrifugation.
† Calculated by adding D < 1.063 and > 1.063 fractions.

lecithin ratios in both fractions were similar to the respective values found in normal subjects (25).

The distribution of free and total cholesterol in the density fractions of these ten patients is shown in Table VI. All of the patients with jaundice showed a relative elevation of free and total cholesterol as well as an absolute elevation of free cholesterol in the fraction D < 1.063. In two of the jaundiced patients (L.U., I.H.), there was also a marked increase in the ratio of free/total cholesterol in the fractions D < 1.063 and > 1.063.

The distribution of the triglyceride in the density fractions of nine of the patients is shown in Table VII. Although the error involved in this determination may be considerable, the patients with jaundice had a relatively greater amount of triglyceride in the fraction D < 1.063 than had the normal subjects or the other patients.

DISCUSSION

The data presented indicate that the presence of jaundice, whether parenchymatous or obstructive in origin, was associated with changes in the serum lipids, including the individual phospholipids, cholesterol, cholesterol ester and triglyceride, and in the serum lipoproteins.

Of the 12 patients with jaundice, 8 showed an elevation in serum lecithin concentration; the 4 patients who did not, likewise showed no elevation in total serum phospholipid concentration, in 2 (P.K., I.H.) probably because of severe parenchymal damage (5) and in 2 (J.E., J.M.) because of mild disease. But the percentage of the total phospholipid analyzed as lecithin was elevated in 11 of the 12 patients. The serum sphingomyelin and "cephalin" concentrations were more variable, the former usually being normal and the latter elevated. The ratio of sphingomyelin/lecithin, however, was lowered in all of the jaundiced patients. Similar results were reported by Petersen (8, 9), who estimated the individual phospholipids by measuring phospholipid fragments after hydrolysis.

The serum lysolecithin concentration appeared to be inversely related to the direct serum bilirubin concentration and was low in all of the patients with marked primary liver disease, being lowest and almost absent in a patient with acute hepatic insufficiency of the chronic alcoholic (23) and coma. The isolation, identification, and quantitation of lysolecithin in human serum have been described previously (21, 22) and confirmed (30, 31). About one-half of the serum lysolecithin, furthermore, appears to be concentrated in a lipoprotein fraction D > 1.21 (32). A decrease in the serum lysolecithin concentration, therefore, might be reflected in a decrease in the amount of lysolecithin in the lipoprotein fraction D > 1.21. Havel, Eder and Bragdon (19) reported that in eight patients with hyperlipoproteinemia, the amount of phospholipid in the fraction D > 1.21 was within the normal range. Furman, Norcia, Fryer and Wamack (33), however, noted that in a patient with biliary cirrhosis and a high lipid phosphorus level, almost no ethanol-ether soluble phosphorus was found in the fraction D > 1.21.

The decrease in the serum lysolecithin concentration in the patients with jaundice could be the result of biliary retention, of abnormal liver func-

tion, or of both. Borgström (34) has shown that the lecithin of bile is converted to lysolecithin in the gastrointestinal tract. The lysolecithin of serum, therefore, may represent that absorbed from the gastrointestinal tract during digestion, and the decreased serum lysolecithin concentration in the patients with jaundice might have been the result of a decreased excretion of bile into the gastrointestinal tract, even though lecithin might have been introduced into the gastrointestinal tract in the diet. If the retention of bile lecithin parallels the serum bilirubin concentration, then the inverse relation between serum lysolecithin concentration and serum bilirubin concentration suggested in Figure 1 would be expected. This explanation for the decrease in serum lysolecithin concentration in patients with jaundice, however, seems unlikely for several reasons. First, the two patients with the obstructive type of jaundice showed normal levels of serum lysolecithin; but their serum bilirubin levels were lower than most of the other patients. Second, two patients with marked cirrhosis, ascites and edema showed decreases in the serum lysolecithin concentration, even though the serum bilirubin concentration was only slightly elevated. Third, three patients with bile fistula, who were losing much if not all of their bile for at least eight days before the serum lipid analysis, showed normal concentrations of serum lysolecithin. It seems likely, therefore, that the liver controls the level of and, as is the case with the other major phospholipids (35, 36) probably synthesizes and breaks down the lysolecithin of serum. Lysolecithinases, in fact, have been found in rat and sheep liver (37, 38). If this is the case, it apparently takes less hepatic damage to lower the level of the serum lysolecithin than that of the other serum phospholipids. The suggestive correlation of the serum lysolecithin concentration with the direct serum bilirubin concentration thus may represent a correlation with the severity of the parenchymal liver disease, as evidenced by the level of the direct serum bilirubin. The decrease in serum lysolecithin concentration, therefore, appears to be a result of decreased liver function, although the possibility still exists that it is also related to biliary retention in some as yet unknown way.

The association of parenchymatous and obstructive jaundice with an increase in the serum concentration of free cholesterol and in the ratio of free/total cholesterol, as well as the apparent direct relationship between the degree of jaundice and these functions, has been noted before (2–5, 10–12). The lack of a comparable degree of elevation in patients with bile fistula suggests that a decrease of bile in the gastrointestinal tract was not responsible for these changes. The serum cholesterol ester concentration, like the serum lysolecithin concentration, appeared to be inversely related to the serum bilirubin concentration and was decreased in one patient with marked cirrhosis without jaundice. These data suggest that, in patients with parenchymatous or obstructive jaundice, the decrease in cholesterol ester concentration, like the decrease in serum lysolecithin concentration, may be the result of parenchymal damage, while the increase in free cholesterol concentration, like the increase in serum lecithin concentration, is a consequence of biliary retention. Nevertheless, the possibility exists that the decrease in the cholesterol ester and lysolecithin concentrations may also be related to biliary retention as, for example, by competition with free cholesterol and lecithin, respectively, for lipoprotein binding sites. The increase in the ratio of serum free/total cholesterol in patients with jaundice of both causes, however, is usually attributed to a failure in the cholesterol esterification process.

The serum triglyceride concentration was elevated in nine of the patients with jaundice. The values obtained, however, may be in considerable error as they were calculated as the difference between the concentrations of total serum nonphosphorus-containing lipid ester and serum cholesterol ester, and evidence indicates that cholesterol ester may not give a quantitative color yield with the lipid ester method used (39–41). Nevertheless, even if cholesterol ester gave no color yield with this method, the triglyceride values would be elevated in half of the patients with jaundice and in all of the patients with bile fistula. All of the sera in the jaundiced patients, except for one from a patient with very mild hepatitis, however, were clear on inspection, possibly because of insufficient elevation of the serum triglyceride concentration (42). Ahrens and Kunkel (10) also noted increased neutral fat concentrations with clear sera in patients with primary biliary cirrhosis, and Albrink, Man and Peters (11) found the serum neutral fat to be almost invariably elevated in pa-

tients with obstructive jaundice. The elevation in the three patients with bile fistula and the lack of elevation in the patients with marked cirrhosis without jaundice suggest that the increased serum triglyceride concentration might have been related to the decreased delivery of bile into the gastrointestinal tract.

Analysis of the lipoprotein fractions [2] separated by ultracentrifugation in five of the patients with jaundice revealed an elevation of the amount of lipid phosphorus in the fraction $D < 1.063$ in all, even when the total serum lipid phosphorus was not elevated, and a decrease in the amount of lipid phosphorus in the fraction $D > 1.063$ in three of the five patients. Similar findings have been reported by others (15–19). The minimal, if any, deviation from the normal in these fractions in the nonjaundiced patients with cirrhosis and with bile fistula suggests that these changes are related to bile retention. The mechanism of these changes, however, is obscure. The ratios of the individual phospholipids, cholesterol and cholesterol ester in the density fractions were changed in the same direction as in whole serum. The sphingomyelin/lecithin ratio, moreover, was considerably higher and the "cephalin"/sphingomyelin ratio lower in the fraction $D < 1.063$ than in $D > 1.063$, as in normal subjects (25), suggesting that the relative binding affinities for these phospholipids by the respective lipoproteins were similar to the normal.

Although it is difficult to dissociate the effects of biliary retention and parenchymal damage, the evidence presented suggests that in patients with jaundice the hyperlipemia, characterized by an increase in the concentrations of lecithin and free cholesterol, is a consequence of the biliary retention. Although the mechanism of such a relationship is unknown, two explanations may be considered. It has been suggested that the hyperlipemia of obstructive jaundice is a consequence of the retention of cholate in the plasma with a resultant trapping of plasma lipids (44–46). This explanation seems unlikely, however, because the amount of bile salt in the serum of patients with jaundice is much smaller than the amounts of lecithin and free cholesterol (47, 48) and apparently most of the bile salt is bound to albumin (49). In addition, the hyperlipemia appears to be rather selective, in that it is largely accounted for by lecithin and free cholesterol. The second explanation seems more reasonable. It is probably more than coincidental that the lipids which account for most of the hyperlipemia in patients with jaundice, i.e., lecithin and free cholesterol, are the same lipids that account for essentially all of the lipid of bile (50–52). Although the liver apparently synthesizes lecithin and free cholesterol, the hyperlipemia could result from an inability of the liver to excrete these lipids into the bile as a result of parenchymal damage or from a regurgitation of the bile lipids into the blood as a result of biliary obstruction. Evidence offered against this hypothesis has been the demonstration of an increased rate of synthesis of plasma cholesterol following bile duct ligation in the rat (53) and of an increased rate of plasma phospholipid synthesis in patients with jaundice and hyperlipemia (54). It is conceivable, however, that the increased rate of synthesis of these lipids may represent an increment in synthesis that ordinarily is excreted into the bile but in jaundiced patients finds its way into the plasma. Other explanations for the hyperlipemia, however, are possible, and selection of the correct one must await further studies.

[2] The fractions $D < 1.063$ and > 1.063 migrate as β- and α-globulin, respectively, on paper electrophoresis (19), while the fraction $D < 1.019$ migrates as α_2 globulin on zone electrophoresis (43).

SUMMARY

The serum lipids, including the individual phospholipids, free cholesterol, cholesterol ester, and triglyceride were analyzed in 12 patients with parenchymatous or obstructive jaundice, in 3 patients with cirrhosis without jaundice, and in 3 patients with bile fistula.

The serum lipids of the patients with parenchymatous or obstructive jaundice showed a characteristic pattern, even when the total serum lipid concentration was not elevated, which included *1*) elevation of the lecithin concentration and, more consistently, of the ratio of lecithin/total phospholipid, *2*) elevation of the free cholesterol concentration and of the ratio of free/total cholesterol, and *3*) elevation of the concentration of the lipoprotein fraction with density (D) < 1.063 and lowering of the concentration of the lipoprotein fraction $D > 1.063$, and more consistently, elevation of the ratio of the former to the latter.

These serum lipid changes were not seen in the nonjaundiced patients with cirrhosis and with bile fistula, except for some elevation of the ratio of free/total cholesterol, suggesting that this pattern was a concomitant of bile retention rather than of parenchymal damage or decrease in the amount of bile delivered to the gastrointestinal tract.

The serum lipids of the lipoprotein fractions separated by ultracentrifugation reflected the changes seen in whole serum and comparison with the corresponding fractions of normal subjects suggested that the relative binding affinities of the lipoproteins for the individual phospholipids were similar to the normal in all of the patients.

The serum lysolecithin concentration in the patients with parenchymatous jaundice appeared to be inversely related to the direct serum bilirubin concentration. The lowered concentration of lysolecithin in two nonjaundiced patients with cirrhosis and the normal concentration in the three patients with bile fistula suggested that the lysolecithin concentration reflected parenchymal function and that the liver controls the level of and may be the source of this phospholipid.

The serum cholesterol ester concentration varied similarly to the serum lysolecithin concentration and likewise may be related to parenchymal function.

The serum triglyceride concentration was elevated in most of the jaundiced patients and in the patients with bile fistula.

It appears that parenchymal liver damage has a depressing effect upon the serum concentrations of the phospholipids, free cholesterol, and cholesterol ester, while biliary retention tends to elevate the serum concentrations of lecithin and free cholesterol.

REFERENCES

1. Flint, A., Jr. Experimental researches into a new excretory function of the liver; consisting in the removal of cholesterine from the blood, and its discharge from the body in the form of stercorine. Amer. J. med. Sci. 1862, **44**, 305.
2. Feigl, J. Neue Untersuchungen über akute gelbe Leberatrophie. III. Fette und Lipoide des Blutes. Chemische Beiträge zur Kenntnis der Entwicklung und Charakteristik spezifischer Lipämien. Biochem. Z. 1918, **86**, 1.
3. Thannhauser, S. J., and Schaber, H. Über die Beziehungen des Gleichgewichtes Cholesterin und Cholesterinester im Blut und Serum zur Leberfunktion. Klin. Wschr. 1926, **5**, 252.
4. Epstein, E. Z. Cholesterol of the blood plasma in hepatic and biliary diseases. Arch. intern. Med. 1932, **50**, 203.
5. Man, E. B., Kartin, B. L., Durlacher, S. H., and Peters, J. P. The lipids of serum and liver in patients with hepatic diseases. J. clin. Invest. 1945, **24**, 623.
6. Thannhauser, S. J., and Magendantz, H. The different clinical groups of xanthomatous diseases; a clinical physiological study of 22 cases. Ann. intern. Med. 1938, **11**, 1662.
7. Albrink, M. J. The choline-containing phospholipids of serum. J. clin. Invest. 1950, **29**, 46.
8. Petersen, V. P. The individual plasma phospholipids in acute hepatitis. Acta med. scand. 1953, **144**, 333.
9. Petersen, V. P. The individual plasma phospholipids in obstructive jaundice. Acta med. scand. 1953, **144**, 345.
10. Ahrens, E. H., Jr., and Kunkel, H. G. The relationship between serum lipids and skin xanthomata in eighteen patients with primary biliary cirrhosis. J. clin. Invest. 1949, **28**, 1565.
11. Albrink, M. J., Man, E. B., and Peters, J. P. Serum lipids in infectious hepatitis and obstructive jaundice. J. clin. Invest. 1950, **29**, 781.
12. Zieve, L. Studies of liver function tests. III. Dependence of percentage cholesterol esters upon the degree of jaundice. J. Lab. clin. Med. 1953, **42**, 134.
13. McGinley, J., Jones, H., and Gofman, J. Lipoproteins and xanthomatous diseases. J. invest. Derm. 1952, **19**, 71.
14. Pierce, F. T., Jr., Kimmel, J. R., and Burns, T. W. Lipoproteins in infectious and serum hepatitis. Metabolism 1954, **3**, 228.
15. Kunkel, H. G., and Slater, R. J. Lipoprotein patterns of serum obtained by zone electrophoresis. J. clin. Invest. 1952, **31**, 677.
16. Nikkilä, E. Studies on the lipid-protein relationships in normal and pathological sera and the effect of heparin on serum lipoproteins. Scand. J. clin. Lab. Invest. 1953, **5**, suppl. 8.
17. Eder, H. A., Russ, E. M., Pritchett, R. A. R., Wilber, M. M., and Barr, D. P. Protein-lipid relationships in human plasma: In biliary cirrhosis, obstructive jaundice, and acute hepatitis. J. clin. Invest. 1955, **34**, 1147.
18. Furman, R. H., Conrad, L. L., and Howard, R. P. A serum lipoprotein pattern characteristic of biliary obstruction, with some comments on "jaundice due to methyltestosterone" (abstract). Circulation 1954, **10**, 586.
19. Havel, R. J., Eder, H. A., and Bragdon, J. H. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J. clin. Invest. 1955, **34**, 1345.
20. Russ, E. M., Raymunt, J., and Barr, D. P. Lipoproteins in primary biliary cirrhosis. J. clin. Invest. 1956, **35**, 133.

21. Phillips, G. B. The isolation of lysolecithin from human serum. Proc. nat. Acad. Sci. (Wash.) 1957, **43**, 566.
22. Phillips, G. B. The isolation and quantitation of the principal phospholipid components of human serum using chromatography on silicic acid. Biochim. biophys. Acta 1958, **29**, 594.
23. Phillips, G. B., and Davidson, C. S. Acute hepatic insufficiency of the chronic alcoholic. Clinical and pathological study. Arch. intern. Med. 1954, **94**, 585.
24. Malloy, H. T., and Evelyn, K. A. The determination of bilirubin with the photoelectric colorimeter. J. biol. Chem. 1937, **119**, 481.
25. Phillips, G. B. The phospholipid composition of human serum lipoprotein fractions separated by ultracentrifugation. J. clin. Invest. 1959, **38**, 489.
26. Fiske, C. H., and Subbarow, Y. The colorimetric determination of phosphorus. J. biol. Chem. 1925, **66**, 375.
27. Schoenheimer, R., and Sperry, W. M. A micromethod for the determination of free and combined cholesterol. J. biol. Chem. 1934, **106**, 745.
28. Sperry, W. M., and Webb, M. A revision of the Schoenheimer-Sperry method for cholesterol determination. J. biol. Chem. 1950, **187**, 97.
29. Rapport, M. M., and Alonzo, N. Photometric determination of fatty acid ester groups in phospholipides. J. biol. Chem. 1955, **217**, 193.
30. Gjone, E., Berry, J. F., and Turner, D. A. Isolation and identification of lysolecithin from lipid extracts of normal human serum. Biochim. biophys. Acta 1959, **34**, 288.
31. Marinetti, G. V., Albrecht, M., Ford, T., and Stotz, E. Analysis of human plasma phosphatides by paper chromatography. Biochim. biophys. Acta 1959, **36**, 4.
32. Phillips, G. B. Lipid composition of human serum lipoprotein fraction with density greater than 1.210. Proc. Soc. exp. Biol. (N. Y.) 1959, **100**, 19.
33. Furman, R. H., Norcia, L. N., Fryer, A. W., and Wamack, B. S. Lipoprotein recovery following ultracentrifugal fractionation at solvent density 1.21 as determined by cholesterol and lipid phosphorus analyses of supernatant and bottom fractions. J. Lab. clin. Med. 1956, **47**, 730.
34. Borgström, B. Studies of the phospholipids of human bile and small intestinal content. Acta chem. scand. 1957, **11**, 749.
35. Fishler, M. C., Entenman, C., Montgomery, M. L., and Chaikoff, I. L. The formation of phospholipid by the hepatectomized dog as measured with radioactive phosphorus. I. The site of formation of plasma phospholipids. J. biol. Chem. 1943, **150**, 47.
36. Entenman, C., Chaikoff, I. L., and Zilversmit, D. B. Removal of plasma phospholipides as a function of the liver: The effect of exclusion of the liver on the turnover rate of plasma phospholipides as measured with radioactive phosphorus. J. biol. Chem. 1946, **166**, 15.
37. Dawson, R. M. C. The phospholipase B of liver. Biochem. J. 1956, **64**, 192.
38. Marinetti, G. V., Erbland, J., Witter, R. F., Petix, J., and Stotz, E. Metabolic pathways of lysolecithin in a soluble rat-liver system. Biochim. biophys. Acta 1958, **30**, 223.
39. Entenman, C. Preparation and determination of higher fatty acids *in* Methods in Enzymology, S. P. Colowick and N. O. Kaplan, Eds. New York, Academic Press, 1957, vol. 3, p. 323.
40. Kuo, P. T., and Carson, J. C. Dietary fats and the diurnal serum triglyceride levels in man. J. clin. Invest. 1959, **38**, 1384.
41. Hack, M. H. A method for the estimation of fatty acid esters. Arch. Biochem. 1955, **58**, 19.
42. Albrink, M. J., Man, E. B., and Peters, J. P. The relation of neutral fat to lactescence of serum. J. clin. Invest. 1955, **34**, 147.
43. Kunkel, H. G., and Trautman, R. The α_2 lipoproteins of human serum. Correlation of ultracentrifugal and electrophoretic properties. J. clin. Invest. 1956, **35**, 641.
44. Friedman, M., and Byers, S. O. Cholic acid: An adequate stimulus for hypercholesteremia in the normal fasting rat. Proc. Soc. exp. Biol. (N. Y.) 1951, **78**, 528.
45. Friedman, M., Byers, S. O., and Rosenman, R. H. The accumulation of serum cholate and its relationship to hypercholesteremia. Science 1952, **115**, 313.
46. Byers, S. O., Friedman, M., Biggs, M. W., and Gunning, B. Observations concerning the production and excretion of cholesterol in mammals. IX. The mechanism of the hypercholesteremic effect of cholic acid. J. exp. Med. 1953, **97**, 511.
47. Rudman, D., and Kendall, F. E. Bile acid content of human serum. I. Serum bile acids in patients with hepatic disease. J. clin. Invest. 1957, **36**, 530.
48. Carey, J. B., Jr. The serum trihydroxy-dihydroxy bile acid ratio in liver and biliary tract disease. J. clin. Invest. 1958, **37**, 1494.
49. Rudman, D., and Kendall, F. E. Bile acid content of human serum. II. The binding of cholanic acids by human plasma proteins. J. clin. Invest. 1957, **36**, 538.
50. Polonovski, M., and Bourrillon, R. Les phospholipides de la bile. Bull. Soc. Chim. biol. (Paris) 1952, **34**, 712.
51. Isaksson, B. On the lipid constituents of normal bile. Acta Soc. Med. upsalien. 1952, **56**, 177.
52. Phillips, G. B. The lipid composition of human bile. Biochim. biophys. Acta 1960, **41**, 361.
53. Frederickson, D. S., Loud, A. V., Hinkelman, B. T., Schneider, H. S., and Frantz, I. D., Jr. The effect of ligation of the common bile duct on cholesterol synthesis in the rat. J. exp. Med. 1954, **99**, 43.
54. Balfour, W. M. Human plasma phospholipid formation: A study made with the aid of radiophosphorus. Gastroenterology 1947, **9**, 686.

ZINC IN HUMAN BLOOD CELLS: NORMAL VALUES AND ABNORMALITIES ASSOCIATED WITH LIVER DISEASE *

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Studies of the biochemistry of zinc have produced results which are widely divergent because of difficulties in methodology. However, techniques have evolved so that general agreement now exists that dithizone methods give reliable and reproducible measurements of zinc in erythrocytes and sera, and that acid extraction of the zinc from these biological materials produces more consistent and accurate results than wet-ashing or dry-ashing methods of preparing specimens for dithizone determination of zinc (1–4). Although normal values for erythrocyte and serum zinc have been established, the zinc content of leukocytes from normal persons using the acid extraction-dithizone method has not been evaluated in the past.

Patients with hepatic cirrhosis have been found to have low serum zinc levels (5, 6), zincuria (7), and decreased concentrations of zinc in liver tissue (6, 7). Vallee, Wacker, Bartholomay and Hoch (7) have postulated that these disturbances of zinc metabolism may be due to inhibition and perhaps to degradation of the zinc metalloenzymes, alcohol and glutamic dehydrogenases, because of excessive ethanol ingestion. However, there has been no direct evidence of altered intracellular zinc levels in patients with liver disease.

The purposes of this study were to extend the application of the acid extraction-dithizone method for the determination of zinc to human leukocytes, to re-examine critically the levels of zinc in blood cells of normal persons, and to investigate the zinc content of the erythrocytes and leukocytes of patients with cirrhosis.

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MATERIALS AND METHODS

All reagents were prepared, glassware cleaned, and blood collected with the special precautions required for trace metal analysis (4). Venous blood (about 30 to 40 ml) was drawn from the subjects in the morning, without regard to fasting or nonfasting state. A small aliquot of blood was taken for routine hematological determinations and the remainder was mixed with about an equal volume of polyvinylpyrrolidone-heparin sedimenting solution. After about 45 minutes the leukocyte-rich supernatant was pipetted off. This leukocyte suspension and a suspension of erythrocytes were washed three times with normal saline, and then diluted with saline so that the erythrocyte suspension contained about 500,000 red blood cells per mm^3 and the leukocyte-rich suspension had approximately 40,000 to 50,000 white blood cells per mm^3. An aliquot of each suspension was then removed for quadruplicate counting of erythrocytes and leukocytes in each suspension with a Coulter counter. Random checks of leukocyte counts were also made in hemocytometers.

Berfenstam's method for the acid extraction of zinc from blood was modified slightly and used routinely (2). For some experiments used to evaluate methods, wet-ashing for zinc extraction, as described by Wolff (3) and by Sandell (8), were also used. The determinations of zinc were made according to the dithizone method of Malmstrom (4), modified by using a 1.00 mg per cent dithizone-carbon tetrachloride solution and reading in a Beckman DU spectrophotometer at 520 mμ. Duplicate zinc-free water blanks and duplicate zinc standards (2 or 3 μg) were processed with each set of samples.

In this dithizone method the zinc standard curves obey Beer-Lambert's law to a concentration of 3.5 μg of zinc per sample. It would be possible, therefore, to read the zinc content of a sample directly from a standard curve. However, because of slight variations in standard curves from day to day, probably due to differences in room temperature and slight variations in the dithizone solution concentrations, the zinc content of each sample was determined by using a calibration constant determined for each set. Duplicate determinations were made on each sample in most cases. The values of all duplicate determinations were averaged, and a single result, cor-

TABLE I

Serial determinations of zinc in 3 ml aliquots of the same erythrocyte-rich suspension

Date	μg Zn/3 ml sample
9/10/59	1.41
9/17/59	1.48
9/21/59	1.43
9/22/59	1.45
9/24/59	1.51
9/30/59	1.45
10/5/59	1.50
10/28/59	1.41
10/29/59	1.53

rected for erythrocyte or leukocyte contamination, is shown for each sample tested.

RESULTS

Evaluation of the method. As a check on the day-to-day reproducibility of results, many 3 ml aliquots of erythrocyte-rich suspensions were prepared at one time and stored. A single sample of such a control lot was randomly introduced and the zinc content determined with a batch of other cell samples from time to time. The results of such a control study are shown in Table I and serve to demonstrate the reliability of the method. In this set of control samples, as in other sets, some of the erythrocyte-rich suspensions were stored in zinc-free water added after the final saline wash, and some in hydrochloric acid-trichloroacetic acid mixture. No significant difference in results was introduced by varying the storage state.

Because of the possibility that the acid extraction method of preparing leukocytes for the dithizone determination might not result in complete extraction of the cellular zinc, several experiments were performed using concurrent wet-ashing and acid extraction methods to prepare aliquots of the same leukocyte-rich cell suspensions. As revealed in Table II, there were no significant differences between the leukocyte zinc values determined by these two methods.

TABLE II

Comparison of zinc levels in leukocyte-rich suspensions as determined using wet-ashing and acid extraction methods

Subj.	Cells	Wet-ashing	Acid extraction
		μg Zn/3 ml WBC-rich suspension	
R. D.	1,111	2.02	1.94
A. B.	1,117	1.28	1.11
R. S.	1,124	1.06	1.10

The calculation of leukocyte zinc would be simplified if the leukocyte-rich sample were completely free of contaminating erythrocytes. Wolff lysed the erythrocytes with 2.5 per cent acetic acid (3) and thus obtained a pure leukocyte preparation. Using the method, we found that most or all of the leukocyte zinc was leached from the cells (Table III). In a single experiment the acidity of the saline wash solution was reduced to pH 3.7 with zinc-free hydrochloric acid, and a single washing with this solution resulted in about 50 per cent loss of leukocyte zinc. When either a 2.5 per cent acetic acid or a 1 per cent saponin solution was used to lyse the erythrocytes, the remaining leukocytes tended to be clumped rather markedly, making accurate enumeration of the cells impossible.

Values in normal persons. In 38 simultaneous determinations of erythrocyte and leukocyte zinc concentrations of 32 normal persons (Table IV), using the acid extraction and dithizone determina-

TABLE III

Data showing effect on zinc of washing leukocyte-rich suspension once with 2.5 per cent acetic acid *

Subj.	Cells	Washed thrice with normal saline		Washed once with 2.5% acetic acid and twice with normal saline		Zinc in supernatant acetic acid wash sol.
		μg Zn/10¹⁰ WBC	μg Zn/3 ml WBC-rich susp.	μg Zn/10¹⁰ WBC	μg Zn/3 ml WBC-rich susp.	μg
W. T.	930	61.5	1.78	0.0	0.00	
W. B.	105	57.6	0.83	0.0	0.05	0.93
R. D.	11,111	64.0	1.94	13.3	0.20	
A. B.	1,116			0.0	0.00	1.60
A. B.	1,117	33.1	1.11	0.0	0.00	
R. S.	1,124	72.2	1.10	6.6	0.08	

* Cells used in these experiments were not from normal persons.

TABLE IV

Normal values for zinc in leukocytes and erythrocytes

Subjects			No. of determinations						
Sex	Age range	No.		Range	Mean	SD	Range	Mean	SD
	yrs			*μg Zn/10¹⁰ RBC*			*μg Zn/10¹⁰ WBC*		
Males	22–41	24	29	9.5–14.4	12.0	1.25	56.8–168	102.0	26.5
Females	25–50	8	9	9.3–15.5	12.2	2.14	57.7–122	105.0	21.5
Both	22–50	32	38	9.3–15.5	12.1	1.51	56.8–168	103.0	25.5

TABLE V

Comparison with normal subjects of the range and mean of values for leukocyte and erythrocyte zinc in 15 cirrhotics, and for serum zinc in 9 cirrhotics

	Normals			Cirrhotics		
	Range	Mean	SD	Range	Mean	SD
μg Zn/10¹⁰ leukocytes	56.8–168.0	103.0	25.5	39.9–106	72.9	19.9
μg Zn/10¹⁰ erythrocytes	9.3– 15.5	12.1	1.51	7.9– 18.0	12.5	3.16
μg Zn/100 ml serum	84.0–163.0	124.0	15.9 (11)	36.0– 80.0	64.2	13.1

tion method, we found a mean of 12.1 μg of zinc per 10^{10} erythrocytes (SD ± 1.51) and 103 μg of zinc per 10^{10} leukocytes (SD ± 25.5).

Values in patients with cirrhosis. The results, which are summarized in Table V, show that the leukocyte zinc values in 15 cirrhotic patients range from 39.9 to 106 μg per 10^{10} white cells with a mean of 72.9 μg per 10^{10} white cells. These values are distinctly lower than normal, and the differences can not be explained on the basis of

TABLE VI

Summary of zinc determinations and of clinical and laboratory data at the time of study for each of 15 patients with hepatic cirrhosis

Patient	Age, race, sex	Bilirubin	BSP*	RBC	WBC	Serum	PCV†	MCV‡	Leukocyte counts§	
									Total	Differential
				μg Zn/10¹⁰	*μg Zn/10¹⁰*	*μg Zn /100 ml*				
W. B.	39 WM‖	13.0		15.1	66.6		42	121	10,850	Normal
W. C.	40 NM‖	0.62	14	11.4	97.0	63	51	89	11,000	83.5% Neutrophils
P. F.	65 WM‖	0.50		12.1	48.1	36	33	82	6,030	Normal
I. G.	65 WM‖		28	8.0	95.2	72	41	102	9,370	Normal
R. H.	38 WM‖	1.66	32	12.7	59.4	68	32	84	7,970	Normal
A. J.	50 WM‖	14.1		15.2	60.3		35	121	35,000	92.5% Neutrophils
I. M.	68 WM‖	15.6	49	15.1	39.9		29	106	15,300	92.5% Neutrophils
A.R.	40 WM‖	25.0		14.9	45.5		29	115	23,000	89.0% Neutrophils
E.R.	49 WM‖	2.28	45	16.4	76.2	80	41	104	9,290	Normal
R.S.	37 WM‖	3.25		11.9	87.8		47	94	7,980	Normal
O.S.	65 WM‖	2.72	67	8.1	60.5	48	48	103	8,230	Normal
C.T.	43 WM‖	0.76	24	7.9	80.5	66	27	78	5,880	Normal
O.W.	48 WM‖	1.98		11.5	10[illegible].0		34	89	5,940	Normal
W.W.	57 WM‖	3.90		18.0	94.2	75	33	112	6,470	Normal
W.J., 1	39 WM¶	0.65	23	8.6	81.6	60	48	89	4,870	Normal
W.J., 2	39 WM¶	0.65	29	8.8	70.8	80	41	87	4,530	Normal

* Per cent retention of sulfobromophthalein 45 minutes after injection.
† Packed cell volume or hematocrit.
‡ Mean corpuscular volume of erythrocytes.
§ Total counts of leukocytes in 1 cu mm of peripheral blood, and differential counts of 200 leukocytes in smear of peripheral blood.
‖ Alcoholic cirrhosis.
¶ Post-hepatitic cirrhosis.

the total or differential leukocyte counts or on any morphological feature of the leukocytes.

The range of erythrocyte zinc in these patients is broader than normal, 7.9 to 18.0 μg per 10^{10} red cells, although the mean value, 12.5 μg per 10^{10} red cells, is not significantly different from the normal mean (Table V). As seen in Table VI, the content of zinc in erythrocytes tends to vary directly with the mean corpuscular volume of erythrocytes.

Ten determinations of serum zinc were made on samples from 9 of the patients with cirrhosis. The values range from 36 to 80 μg with a mean of 64.2 μg of zinc per 100 ml serum for the 9 patients.

DISCUSSION

The erythrocyte zinc levels reported here agree closely with those reported by other investigators (1–3, 9). However, the zinc levels of normal leukocytes which are presented here vary greatly from those previously reported, being only about 30 to 50 per cent of the values given by Vallee and Gibson (9), who used a dry-ashing process, and by Wolff (10), who used a wet-ashing method (Table VII).

Vallee and Gibson's zinc values for normal leukocytes were calculated on the basis of the assumption that 100 per cent recovery of uncontaminated leukocytes from the peripheral blood was obtained, i.e., the actual number of leukocytes in each sample was not determined directly, and there is a possibility that recoveries were incomplete. It should also be mentioned that the values for serum zinc reported by Vallee and Gibson (9) and by Hoch and Vallee (1) have been "corrected" downward about 50 per cent in subsequent work by Vikbladh (11), Berfenstam (2), Wolff (10), and Vallee and co-workers (5). It is possible that the same unknown technical difficulty that resulted in the erroneously high serum zinc values also contributed to the high values for zinc in normal leukocytes reported by Vallee and Gibson.

TABLE VII

Previously reported values for zinc in leukocytes of normal persons

Author	Subjects	Range	Mean
		μg Zn/10^{10} WBC	
Vallee and Gibson (9)	17 males	120–650	290
Vallee and Gibson (9)	18 females	170–620	350
Wolff (10)	24 males	152–348	225
Wolff (10)	20 females	160–332	226

Wolff's zinc values for normal leukocytes were determined from leukocyte-rich samples which had been purified by lysing the erythrocytes with 2.5 per cent acetic acid wash solution. As mentioned earlier in this paper, this process apparently leaches the zinc from leukocytes and also makes sampling and counting of the cells very difficult.

Our acid extraction method was compared with the wet-ashing method of Wolff, and the values for zinc in leukocytes by the two methods were in good agreement. The methods used in this study were not evaluated in reference to the dry-ashing method of liberating zinc from biological materials, since dry-ashing has been shown by skilled investigators to be more subject to errors due to contamination and losses (2, 4, 10).

The previously reported evidence for disturbed zinc metabolism in patients with liver disease is corroborated by the finding of low serum zinc levels in our group of cirrhotics. Our mean of 64.2 (SD ± 13.1) μg of zinc per 100 ml serum is similar to the 66 (SD ± 19) μg of zinc per 100 ml serum in 28 patients with advanced cirrhosis described by Vallee and colleagues (5), and the 64.4 (SD ± 19.0) μg zinc per 100 ml serum in 12 cirrhotics reported by Rechenberger (6).

The definitely decreased levels of zinc in the leukocytes of our patients with cirrhosis afford the first direct evidence that the disturbance of zinc metabolism in cirrhosis is reflected by changes of intracellular zinc. Knowledge concerning the zinc in leukocytes is limited. A zinc metalloprotein, estimated to account for 80 per cent of their zinc, has been described, but no biochemical function has been ascribed to it (12). The activity in leukocytes of carboxypeptidase and certain dehydrogenases, which are known to be zinc metalloenzymes in other animal and plant tissues, does not correlate with the zinc content of human white cells (12). Leukocyte alkaline phosphatase is also thought to be a zinc metalloenzyme (13), but studies in this laboratory have not shown any relationship between leukocyte alkaline phosphatase activity and leukocyte zinc values. Indeed, no correlation of zinc levels in leukocytes with any enzy-

matic or other biochemical aberrations has yet been established. However, it seems most reasonable to suspect that the significance of abnormalities in zinc levels is dependent upon a zinc metalloenzyme. Correlative trace metal analysis and enzymatic studies of the easily biopsied leukocytes offer a likely route to the understanding of the biochemical and physiological roles of zinc in the human organism.

Our most advanced knowledge concerning zinc in humans relates to erythrocytes. There is excellent evidence that the level of zinc is directly related to the activity and the content of the zinc metalloenzyme, carbonic anhydrase, in the red cells (10, 14, 15). Therefore, the erythrocyte zinc values for the cirrhotic patients might be assumed to correlate with their carbonic anhydrase activity, but they can not be correlated with the degree of anemia, with the severity of the illness as judged by clinical or biochemical findings, or with the serum or leukocyte zinc values. These sub- to supra-normal levels of red cell zinc tend to correlate with the mean corpuscular volumes of the erythrocyte samples. A marked elevation of zinc per erythrocyte in pernicious anemia has been demonstrated by several investigators (10, 14–17), but the elevation in this disease exceeds the level that might be anticipated on the basis of macrocytosis alone. It has been stated that the unit red cell zinc is normal in other anemias, with the possible exception of sickle cell anemia (16). Since this study has not shown a conclusive correlation of unit erythrocyte zinc to cell size, further study is necessary to clarify this relationship.

SUMMARY

1. An acid extraction method of liberating zinc for determination by a dithizone method has been applied to the study of leukocytes, and evidence for its reliability has been offered.

2. Normal values for zinc in human leukocytes and erythrocytes, using the method, are reported.

3. A decrease of serum zinc concentration in cirrhosis is confirmed, and the evidence for disturbed zinc metabolism in these cases is extended by the finding of decreased intracellular zinc in the leukocytes of these patients.

4. Erythrocyte zinc ranges from below to above normal in the group of cirrhotics, apparently varying directly with mean corpuscular volume.

5. The biochemical and physiological significance of zinc in leukocytes and of the disturbed zinc metabolism in patients with cirrhosis remains to be defined.

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REFERENCES

1. Hoch, F. L., and Vallee, B. L. Precipitation by trichloroacetic acid as a simplification in the determination of zinc in blood and its components. J. biol. Chem. 1949, **181**, 295.
2. Berfenstam, R. Studies on blood zinc: A clinical and experimental investigation into the zinc content of plasma and blood corpuscles with special reference to infancy. Acta paediat. (Uppsala) 1952, **41**, suppl. 87.
3. Wolff, H. Untersuchungen über die Bestimmung von Zink mit Dithizon in biologischen Präparaten. Biochem. Z. 1954, **325**, 267.
4. Malmstrom, B. G. Determinations of zinc in biological materials *in* Methods of Biochemical Analysis, D. Glick, Ed. New York–London, Interscience Publishers, 1956, vol. 3, p. 327.
5. Vallee, B. L., Wacker, W. E. C., Bartholomay, A. F., and Robin, E. D. Zinc metabolism in hepatic dysfunction. I. Serum zinc concentrations in Laënnec's cirrhosis and their validation by sequential analysis. New Engl. J. Med. 1956, **255**, 403.
6. Rechenberger, J. Beitrag zum Zinkstoffwechsel bei der Leberzirrhose. Dtsch. Z. Verdau.-u. Stoffwechselkr. 1958, **18**, 173.
7. Vallee, B. L., Wacker, W. E. C., Bartholomay, A. F., and Hoch, F. L. Zinc metabolism in hepatic dysfunction. II. Correlation of metabolic patterns with biochemical findings. New Engl. J. Med. 1957, **257**, 1055.
8. Sandell, E. B. Colorimetric determination of traces of metals *in* Chemical Analysis, B. L. Clarke and I. M. Kolthoff, Eds. New York–London, Interscience Publishers, 1959, vol. 3, 3rd ed., p. 552.
9. Vallee, B. L., and Gibson, J. G., II. The zinc content of normal human whole blood, plasma, leucocytes, and erythrocytes. J. biol. Chem. 1948, **176**, 445.
10. Wolff, H. P. Untersuchungen zur Pathophysiologie des Zinkstoffwechsels. Klin. Wschr. 1956, **34**, 409.
11. Vikbladh, I. Studies on zinc in blood. Scand. J. clin. Lab. Invest. 1951, suppl. 2.
12. Vallee, B. L. Biochemistry, physiology and pathology of zinc. Physiol. Rev. 1959, **39**, 443.

13. Trubowitz, S. Isolation, purification, properties of alkaline phosphatase from human leukocytes (abstract). Blood 1960, **15**, 419.

14. Vallee, B. L., and Altschule, M. D. "Trace" metals in blood, with particular reference to zinc and carbonic anhydrase. Blood 1949, **4**, 398.

15. Vallee, B. L., Lewis, H. D., Altschule, M. D., and Gibson, J. G., II. The relationship between carbonic anhydrase activity and zinc content of erythrocytes in normal, in anemic and other pathologic conditions. Blood 1949, **4**, 467.

16. Vallee, B. L., and Gibson, J. G., II. The zinc content of whole blood, plasma, leukocytes and erythrocytes in the anemias. Blood 1949, **4**, 455.

17. Talbot, T. R., Jr., and Ross, J. F. The zinc content of plasma and erythrocytes of patients with pernicious anemia, sickle cell anemia, polycythemia vera, leukemia, and neoplastic disease. Lab. Invest. 1960, **9**, 174.

X-RAY DIFFRACTION STUDIES OF HUMAN AORTIC ELASTIN RESIDUES *

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Abnormal calcification is readily identified both grossly and microscopically in the atherosclerotic plaques of aging human aortic intima (1). Concurrently, and frequently independently, considerable amounts of calcium are deposited in the media. This deposition need not occur in regions of prior lipid deposition (2); indeed, even in the absence of atherosclerosis, the calcium content of aorta has been found to increase with age (3).

Such calcific deposits, mainly from atherosclerotic plaques, have been studied by Carlström, Engfeldt, Engström and Ringertz (4), who tentatively identified the crystallites as hydroxyapatite ($Ca_{10}(PO_4)_6(OH_2)$). Their studies have indicated that the X-ray diffraction powder pattern of grossly visible plaques is essentially similar to that given by bone. Further studies have indicated that the dry mass of human arterial wall is greatest in the media, at a region where elastin is the predominant fiber (5). It appeared possible, therefore, that the hydroxyapatite crystallites might be associated with aortic elastin residue (2).

Elastin is generally separated from the other proteins of aorta by virtue of its marked insolubility in dilute alkali (6), formic acid (7) or hot water (8). Each of these methods has been used to separate elastin residues of relatively similar properties and amino acid composition, although the relationship these products bear to the native protein is by no means clear (7). Such products have been studied by chemical analysis (2) and electron microscopy (9), recently aided by the solubilization of elastin by a relatively specific pancreatic enzyme, elastase (10). The current study was undertaken to demonstrate the presence of crystallites in human aortic elastin residues (AER) by means of X-ray diffraction analysis. Elastin was prepared from aortas obtained at necropsy from individuals aged 8 to 86. In order to demonstrate the possible association of hydroxyapatite with elastin rather than with other connective tissue elements, a form of extraction was employed which would separate elastin on the basis of its insolubility in water at autoclave temperatures.

MATERIALS AND METHODS

Preparation of aortic elastin residues.[1] Human aortas were obtained within 24 hours after death from the Office of the Chief Medical Examiner, New York City. Only samples free of moderate to marked intimal plaque formation were employed. The intima-media portion was stripped from the adventitia as in the preparations of Kirk and Dyrbye (11) at 4° C. Immediately thereafter, this portion (from aortic root to bifurcation of iliac vessels) was diced into 0.5 cm^3 segments, washed 3 times with ice cold physiologic saline (0.9 per cent) by centrifugation in the cold, blotted dry until no further moisture could be observed on Whatman no. 1 filter paper, and weighed. This was designated the *wet weight.* Next, these wet aortic slices (6.0 g) were placed in absolute ethanol (250 ml) and homogenized in the baffled flask (500 ml) of a VirTis 45 homogenizer for 10 minutes at top speed (rated at 45,000 rpm) employing the twin-blade attachment. An ice bath kept the temperature below 10° C. The disintegrated aorta and ethanol were centrifuged at 2,000 rpm for 20 minutes, and the residue washed 3 times each with absolute ethanol and ethyl ether, then dried *in vacuo* over $CaCl_2$ to a constant weight designated as the *dry weight.* (During this pro-

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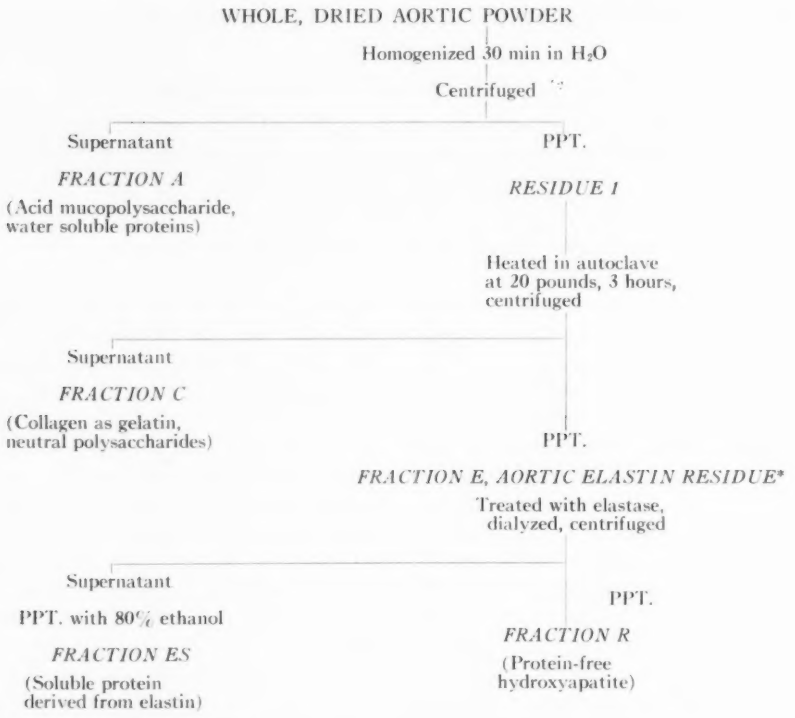
† Present address: Strangeways Research Laboratory, Cambridge, England. Part of this work was done while the author was a Fellow of the Arthritis and Rheumatism Foundation (1958–1959).

[1] The term "aortic elastin residue" is employed to distinguish the final product from that obtained by Lansing (2). In the present study, sections of intima as well as of media have been employed to include crystallites associated with the elastic membranes of aortic *intima* in the material finally examined by X-ray analysis.



* Used in most X-ray diffraction analyses.

FIG. 1. OUTLINE OF PROCEDURE USED IN PREPARING AORTIC ELASTIN RESIDUES (AER, SEE TEXT).

cedure, designed to dry and defat the specimens, a yellow color was imparted to the ethanol during homogenization, and to the first two ethanol washes.) Designated as *dried aortic powder*, this product, stored at room temperature, was used for further fractionation as late as one year after preparation. To determine whether differences in the nature of aortic elastin residues would appear between dried and fresh aortas, in several early experiments the wet aortic slices (6.0 g) were placed directly in the homogenizer with distilled water (280 ml). They were then immediately carried through the fractionation procedure described below.

Further fractionation[2] of powdered aorta was carried out after portions had been allowed to stand with distilled water (2.0 g per 80 ml) at 4° C for 16 hours to rehydrate (Figure 1). Dried, rehydrated aortic powder (2.0 g) was added to distilled water (280 ml) in the baffled flask (500 ml) of the VirTis 45 homogenizer, which was run at top speed for 30 minutes in a room at 4° C. Constant replenishment of an ice bath kept the temperature of the homogenate below 10° C. This procedure yielded an opaque mixture to which ethanol (560 ml) was added. All subsequent procedures were performed at room temperature (23–25° C). Following centrifugation at 2,000 rpm for 45 minutes, the supernatant solution was filtered through glass wool, and potassium acetate (8.5 g) was added to precipitate polyanionic material (12). The precipitate was washed 3 times each with absolute ethanol and ethyl ether and dried *in vacuo* over $CaCl_2$. Judged by its metachromasia with toluidine blue, and precipitability by polyvalent cobalt salts (13), this product was presumed to contain water soluble acid mucopolysaccharide-protein complexes. Chemical analysis of 10 samples of this fraction (Fraction A, Figure 1) gave values, as per cent, within the ranges: hexosamine 4.5 ± 0.5, nitrogen 11.2 ± 0.8, hydroxyproline 0.0.

The *residue* (Residue 1, Figure 1) was washed 3 times each with ethanol and ether and dried as above. When the weights of Fraction A and the elastin-containing Residue 1 were added, the yield was found to be 87 to 96 per cent. The proportion of the two fractions varied somewhat with age.

The dried *residue* was placed in distilled water (20 ml per g) and heated in an autoclave at 20 pounds for 3 hours, maintaining a temperature of approximately 300° C. Two vol of water were added to the resultant gray suspension which was then centrifuged at 2,000 rpm for 40 minutes. The supernatant solution was filtered, evapo-

[2] For sake of clarity, the fractions containing the final product (AER) have been italicized.

rated over steam to one-fifth the original volume, precipitated with two vol of ethanol, and dried and weighed in the above manner. Several samples of this material, designated Fraction C (Figure 1) yielded upon chemical analysis the following, as per cent: hexosamine 2.4 ± 0.6, nitrogen 13.1 ± 0.8, hydroxyproline 6.9 ± 0.5. Based on the high hydroxyproline and hexosamine concentrations, this fraction probably contained collagen that had been converted to gelatin, and neutral polysaccharides. The *residue* was washed 3 times each with alcohol and ether, and dried as before. This product, designated Fraction E (Figure 1) represented AER. Fractions C and E were obtained in yields of 78 to 92 per cent from Residue 1. AER appeared as a fine white or beige powder which could conveniently be used for X-ray diffraction analysis. The hydroxyproline content of several samples was 1.3 ± 0.3 per cent. While not strictly comparable with elastin prepared by alkali or formic acid treatment, this product most closely resembled the material studied by Partridge, Davis and Adair (8).

Treatment with elastase. It has been shown that treatment of elastin with elastase results in the formation of soluble proteins (14). To demonstrate the intimate association of hydroxyapatite crystallites with elastin, samples of AER were treated with this enzyme. Were such association to exist, the finding of a hydroxyapatite X-ray diffraction pattern would be expected in the ethanol-precipitated, nondialyzable supernatant solution of elastase-treated aortic residue. In such an experiment, two samples (100 mg) of aortic elastin residue (A, 55–39) were incubated in a 15 ml Erlenmeyer flask with commercially obtained, crystalline elastase[3] (20 mg) previously dissolved in 5.0 ml of barbital buffer, pH 8.6, 0.1 M. Two similar samples without enzyme added to buffer served as controls. After incubation for 18 hours at 37° C in a constant-temperature water bath, the contents of the four incubation vessels were transferred quantitatively to dialysis bags (Visking, 1⅛ inch) with 5 ml of additional buffer. The contents of the bags were dialyzed against distilled water (6 L) with constant stirring for 24 hours at 4° C. Thereafter, the contents were transferred to centrifuge tubes (25 ml), made up to 15 ml with distilled water, and spun at 3,000 rpm for 45 minutes. All residues were washed 3 times each with alcohol and ether and dried as before. All supernatant solutions representing that protein which had been made soluble, but nondialyzable, by such treatment had ethanol added to 80 per cent vol/vol. After addition of 1 per cent KAc, they were allowed to stand for 3 hours at 4° C. The fine precipitates, which formed only in the tubes containing elastase-treated material, were washed and dried as above. It was possible that incomplete centrifugation might leave crystallites in the nondialyzable supernatant solutions of elastase-treated AER. The appearance of crystals in the ethanol-precipitated supernatant solutions, as disclosed by X-ray diffraction analysis, would in such case *not* be due to the specific action of elastase, but to a nonspecific precipitation of crystallites by any protein in the presence of ethanol. To exclude this possibility, 1.0 ml of a 1 per cent human serum albumin (American Red Cross) was added to the dialyzed supernatant solution of similar nonelastase-treated control samples. Following the addition of albumin, alcohol was added with KAc as above, and the precipitate which formed immediately was washed and dried as before. All samples were subjected to X-ray diffraction analysis by the powder method.

[3] Nutritional Biochemicals, Cleveland, Ohio.

X-ray diffraction. X-ray diffraction studies were performed in a Debye-Scherrer powder camera with a 57.3 mm radius. The powdered residue was placed in 0.2 mm Lindermann glass capillary tubes and exposed for 20 hours. Copper radiation (CuKα = 1.54 Å), with a nickel filter, and Kodak no-screen X-ray film were used. The patterns were analyzed by measuring the diffraction angles θ corresponding to various interatomic spacings of the crystalline sample. The interatomic spacings d were computed from the Bragg equation

$$n\lambda = 2\ d\ \sin\ \theta$$

where n is a whole number indicating the order of reflections and λ is the wavelength of the X-rays expressed in Å units. The intensities of the diffraction lines were visually estimated and the crystalline diffraction pattern was identified with the aid of the X-ray Powder Data File (formerly known as the A.S.T.M. Card Index System).

It should be emphasized that a typical, sharp diffraction pattern is obtained by the powder method if the crystallites of the sample are not too small (above 1,000 Å) and exhibit a regular three-dimensional periodicity of their atomic structure. Proteins, because of their large and irregular periodicity, and amorphous materials, because of the absence of three-dimensional periodicity, do not give rise to conventional powder diffraction patterns. These materials exhibit only diffuse halos near the primary beam.

Analyses. Nitrogen was determined by the Kjeldahl method, hexosamine by the method of Schloss (15). Hydroxyproline was determined by the method of Troll and Cannan (16). Elemental analyses for calcium and carbon were performed by Dr. Carl Tiedcke of Teaneck, N. J.

RESULTS

Analytic and X-ray studies of whole aortic residues. The analytic and gravimetric data obtained from nine samples of AER are listed in Table I. The last two columns indicate a visual estimate of the relative amounts of protein and crystalline matter observed by X-ray diffraction analysis. Dry weight of the samples was relatively constant, averaging 24 per cent of the wet weight, and did not vary appreciably with age. There was a trend toward a weight increase in AER with advancing age. It is apparent that while the nitrogen content decreased with advancing age, the per-

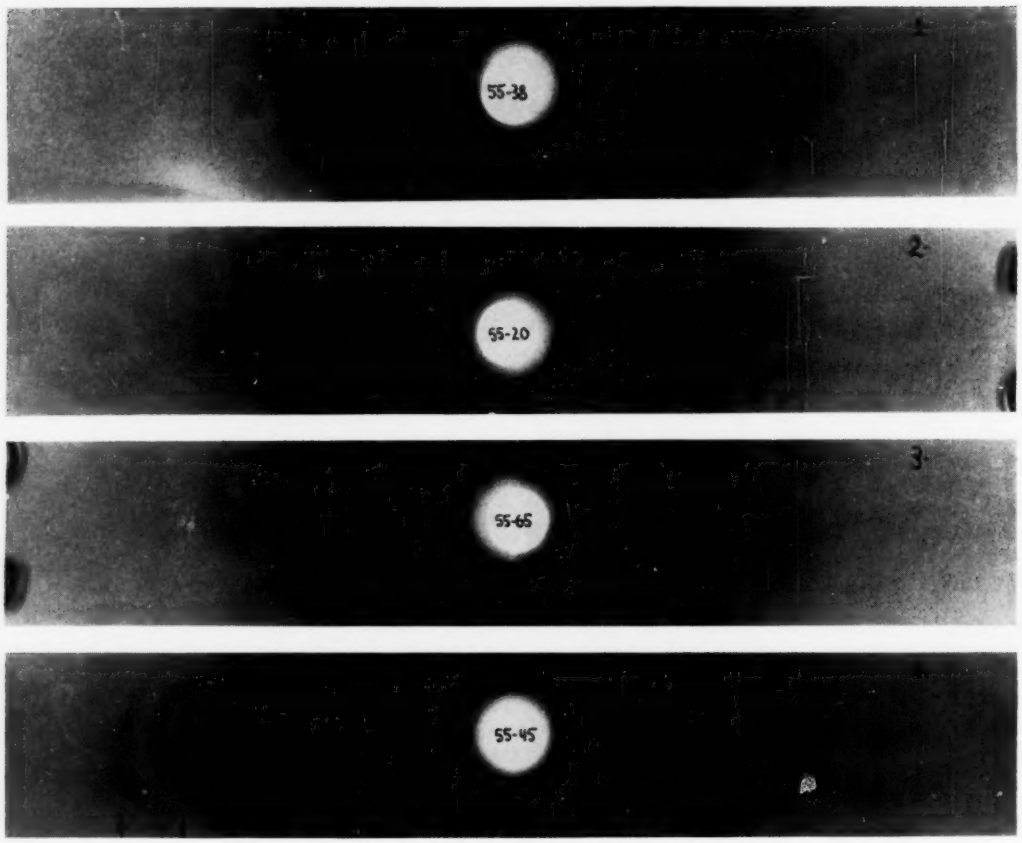


FIG. 2. X-RAY DIFFRACTION POWDER PATTERNS OF HUMAN AORTIC ELASTIN RESIDUES. (CuKα radiation, filter, 20 hours' exposure.) 1, Age 8 years. 2, Age 30. 3, Age 37. 4, Age 41 [arrows indicate the faint crystalline pattern of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$]. 5, Age 80, 24 hour exposure. 6, Age 86, prepared from fresh aorta (see text). 7, Identical with 6, but with superimposed single crystal patterns which could not be identified as hydroxyapatite. 8, Age 86, prepared from dried aortic powder (see text).

centage of calcium rose markedly. Similarly, the carbon content varied inversely with age.

AER obtained from individuals aged 8, 28, 30 and 37 years did not exhibit crystalline diffraction patterns but showed strong, diffuse haloes characteristic of a high protein content (Figure 2, nos. 1–3). This finding was in good agreement with the high nitrogen and carbon content of these samples. The first crystalline pattern was given by the elastin residue extracted from the aorta of a 41 year old woman (Figure 2, no. 4). Despite the strong, diffuse scattering associated with the high protein content of the specimen, a diffraction pattern emerged which could be identified as hydroxyapatite-like. (The techniques employed are not capable of distinguishing unequivocally between hydroxyapatite and closely related apatites and their solid solutions. For instance, fluorapatite and tribasic calcium phosphate give closely similar patterns, but the former may be excluded from human material, and patterns given by tribasic calcium phosphate agree less well with the observed data in Table II. For these reasons, the observed pattern will be referred to as hydroxyapatite.)

In elastin residues prepared from the aortas of individuals aged 63, 80 and 86, the diffuse protein scattering was definitely decreased. Con-

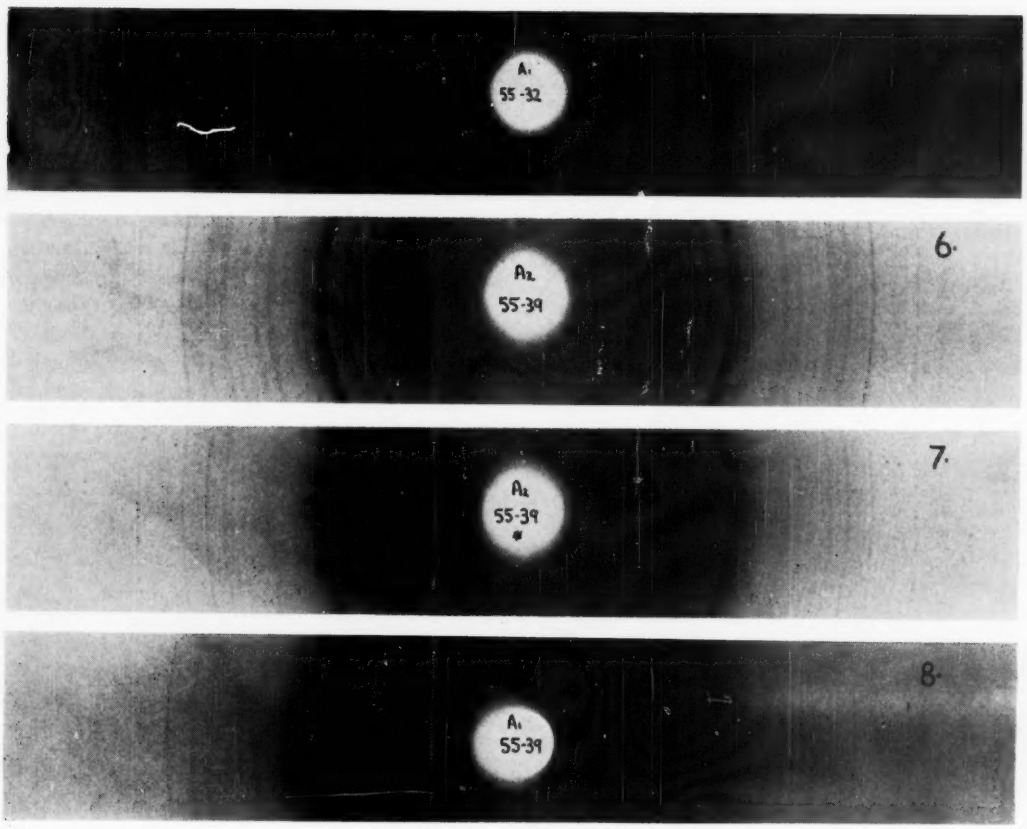


FIG. 2.—*Continued*

TABLE I

Analytic and gravimetric data for nine isolated human aortic residues *

Sample	Age	Sex	Whole aorta	Elastin residue	Analytic values of elastin				X-ray diffraction pattern	
					Nitrogen	Calcium	Carbon	Hexosamine	Protein	Crystal
			% dry weight		%	%	%	%		
55–15	8	M	25.0	54.0	16.3	0.8	49.6		++++	0
55–38	28	M	25.5	48.3	16.8	0.1	49.6		++++	0
55–20	30	M	24.1	49.4	15.8	2.8	47.5	0.2	++++	0
55–65	37	F	24.2	49.0	15.4			0.1	++++	0
55–45	41	F	20.0	53.0	14.0	17.3	48.7	0.1	+++	+
55–46	63	M	23.5	62.9	13.9	10.5	41.5	0.6	++	++
55–32	80	F	28.1	59.0	8.4	31.4	25.5		+	+++
A_1 55–39	86	F	21.0	62.9	9.3	28.2	29.1	0.4	+	+++
A_2 55–39†	86	F	21.0	68.4	10.8	22.4	32.3	0.6	+	++++‡

* The last two columns refer to the relative strength of protein and hydroxyapatite patterns as seen in X-ray diffraction patterns. These are graded from 0 (absent) to ++++ (marked) and were estimated visually (see Figures 2 and 3).

† Prepared directly from sample 55–39 without prior drying.

‡ Single crystal patterns superimposed upon diffraction lines.

TABLE II

d-Spacings calculated from X-ray diffraction analysis (Powder method) of isolated human aortic elastin residues*

hkl	Hydroxyapatite† (Hexagonal $a_0=9.42$ $c_0=6.88$)	Human aortic elastin residues							
		$A_2$55–39	$A_1$55–39	$A_1$55–52	55–45	$A_1$55–39	$A_1$55–39 Fraction D	$A_1$55–39 Fraction R	$A_1$55–39 Fraction ES
100	8.17 VW	7.09	8.38 VW	8.14 VW		8.17 VW	8.28	8.22 VW	
101	5.26 VW	5.18 VW	5.18 W	5.14 M		5.12 VW	5.08	5.09 VW	
200	4.07 VW	4.14 VW				3.99 VW			
111	3.88 VW			3.93 VW		3.85 VW			
201	3.51 VW	3.57 S		3.54 S					
002	3.44 W	3.46 S	3.42 S	3.42 S		3.44 S	3.42 S	3.46 M	3.42 S
102	3.17 VW	3.16 W	3.17 W	3.17 S		3.16 VW	3.15 VW	3.14 VW	3.18 W
210	3.08 VW	3.07 VW	3.11 VW					3.09 VW	
211	2.81 VS	2.84 W	2.85 M	2.86 VS	2.84 VS	2.84 M	2.84 S	2.84 M	2.87 S
112	2.78 S	2.79 VS	2.78 VS			2.79 VS	2.79 VS	2.80 VS	2.79 VS
300	2.72 M	2.71 W	2.71 S			2.74 S	2.70 S	2.73 S	2.71 M
202	2.63 VW	2.63 VW	2.63 VW				2.63 VW	2.63 VW	2.64 VW
301	2.53 VW	2.49 VW	2.53 VW	2.53 M	2.57 VW	2.57 VW	2.59 VW	2.58 VW	2.58 VW
310	2.26 W	2.25 VW	2.25 VW	2.24 VW		2.27 W	2.24 W	2.27 VW	
311	2.15 VW	2.13 VW	2.15 VW	2.15 VW		2.16 VW	2.14 W	2.16 VW	
113	2.07 VW		2.06 VW			2.07 VW	2.06 W	2.07 VW	
303	2.00 VW		2.01 VW	2.00 VW		2.00 VW		2.00 VW	
222	1.94 W	1.95 W	1.94 W		1.98 W	1.94 W	1.94 M	1.95 W	1.94 VW
312	1.89 VW		1.91 VW	1.91 VW		1.91 VW		1.89 VW	
213	1.84 W	1.85 W	1.84 W	1.88 VW		1.84 W	1.82 M		1.84 VW
411, 004	1.72 W	1.72 W	1.72 W	1.72 W		1.71 W		1.70 W	1.72 VW
313	1.61 VW		1.65 VW	1.65 VW	1.60	1.60 VW		1.61 VW	
501	1.59 VW		1.56 VW					1.58 W	
331	1.53 VW	1.54 VW		1.53 W	1.54 W	1.54 VW	1.54 W		

* Intensities estimated visually: very strong (VS) = 100, strong (S) = 70–90, medium (M) = 50–70, weak (W) = 30–50, very weak (VW) = <20.

† X-Ray Power Data File Card no. 9–342 (prepared by P. De Wolff), Techn. Phys. Dientst., Delft, Holland, 1959.

comitantly, the diffraction pattern of hydroxyapatite became more conspicuous (Figure 2, nos. 5–8).

The *d* spacings and corresponding intensities of all samples analyzed are given in Table II. These are compared with a standard diffraction pattern of hydroxyapatite from the X-ray powder data file. It will be seen that virtually all lines are in close agreement with those of the standard pattern. The slight deviations in *d* spacings observed in some of the samples may indicate the presence of solid solutions of hydroxyapatite as postulated by Posner and Stephenson (17).

One preparation derived from an 86 year old woman, (Figure 2, no. 7) exhibited, superimposed upon the powder pattern of apatite, diffraction patterns characteristic of single crystals. The coincidence of the single crystal diffraction spots with lines not belonging to the hydroxyapatite powder pattern indicated that they were of different, not yet identified, origin. All samples that exhibited the diffraction pattern of hydroxyapatite had a considerable calcium content. Since, as indicated above, X-ray diffraction analysis can only indicate the mineral content present in crystalline form, it was not unexpected that no direct correlation could be observed between the total calcium content determined chemically and the intensity of the diffraction lines (compare Tables I and II).

Treatment of human aortic elastin residue with elastase. Treatment of AER with elastase yielded two nondialyzable fractions. Fraction R was a finely powdered, white precipitate with approximately twice the calcium and one-third the nitrogen (in per cent) of the original sample (Table III). X-ray diffraction analysis of this fraction exhibited the highly resolved diffraction pattern shown in Figure 3, no. 10, which was virtually free of the diffuse scattering associated with the presence of protein.

Fraction ES was formed by ethanol precipitation of the nondialyzable supernatant solution resulting from elastase treatment of AER. This material was flocculent and finally fibrous, but en-

tirely white, whereas the original residue was slightly beige. When subjected to X-ray diffraction analysis, in addition to the expected protein scattering, Fraction ES showed a pattern readily identified as hydroxyapatite (Figure 3, no. 11). In order to demonstrate that the hydroxyapatite crystallites in Fraction ES were bound to protein, it was necessary to show that they would not remain in the supernatant fraction if they were unassociated with nondialyzable material. Separation might have occurred if larger crystallites were to precipitate during centrifugation, leaving the finer crystallites to float in the supernatant. Consequently, a diffraction pattern was obtained *without* rotating the specimen. It may be seen in Figure 3, no. 11a, that the lines are just as continuous as in the rotated specimen. This indicated that the crystallite size was of the order of magnitude of 0.5 to 5 μ. Larger crystallites would have given distinct spots of single crystals. Fraction R also yielded a diffraction pattern with continuous lines when the specimen was examined without rotation. This would imply that supernatant and precipitate were not separated on the basis of crystallite size.

Control samples carried through this procedure, save for the substitution of inert buffer for elastase, yielded only one fraction (Fraction D, Tables II and III). This fraction was essentially identical with that of the starting material by chemical and

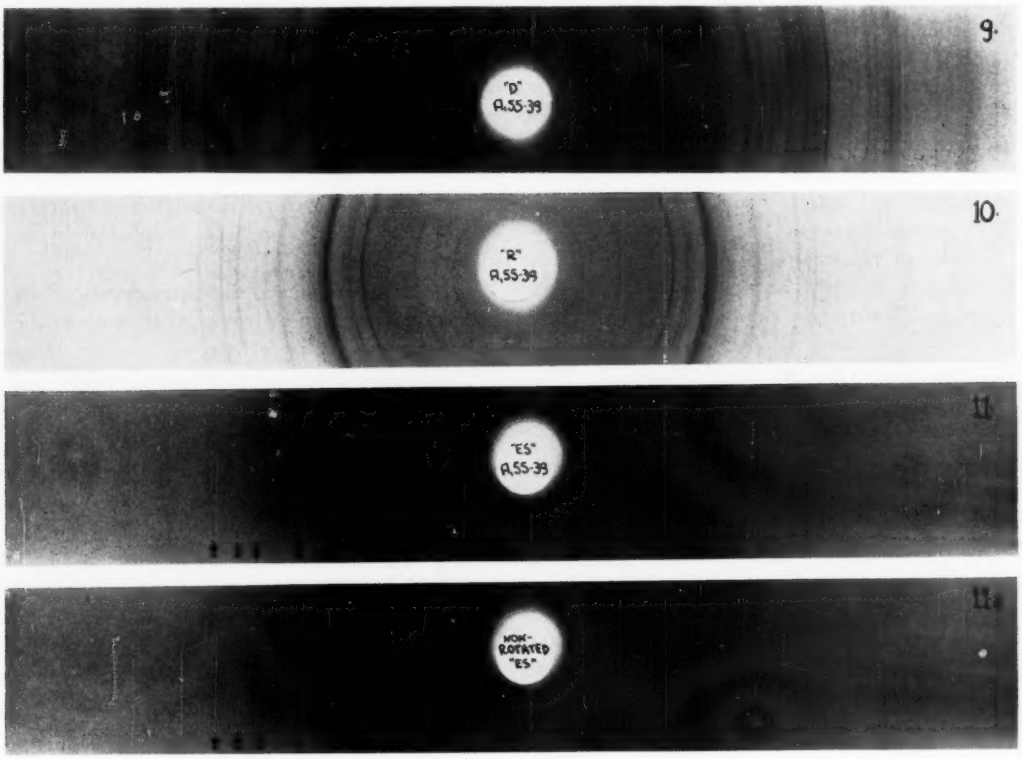


FIG. 3. X-RAY DIFFRACTION POWDER PATTERNS OF HUMAN AORTIC RESIDUES. (CuKα radiation, nickel filter, 20 hrs. exposure.) 9, Elastin residue (A, 55–39) incubated in barbital buffer and dialyzed (Fraction D, Figure 1). 10, Insoluble precipitate of another sample of A, 55–39 after elastase digestion and subsequent dialysis. Note the disappearance of the diffuse diffraction halo associated with protein (Fraction R, Figure 1). 11, Ethanol-precipitated, nondialyzable supernatant of sample A, 55–39 after elastase digestion. Note the characteristic hydroxyapatite pattern, indicated by arrows, and the prominent diffraction halo associated with protein (Fraction ES, Figure 1). 11a, Identical sample as 3. Pattern obtained *without* rotation of specimen. The continuous lines indicate that the crystallite size of the sample is between 0.5 and 5 μ.

TABLE III

Treatment of aortic elastin residue with elastase (sample $A_1$55–39) *

Sample	Symbol	Yield elastin	Calcium	Nitrogen	Carbon	X-ray diffraction pattern	
						Protein	Crystal
		mg/100 mg	%	%	%		
Original elastin	$A_1$55–39		28.2	9.3	29.1	+	+++
Dialyzed elastin	D	89.8	24.5	10.2	31.0	+	+++
Elastase-treated, nondialyzable residue	R	23.1	53.9	3.1	9.8	0	++++
Elastase-treated, nondialyzable supernatant	ES	5.3				++++	+

* After dialysis of the digestion products, centrifugation yielded the fractions R and ES; Fraction ES was reprecipitated with 80% vol/vol ethanol from the supernatant of such a dialyzed digestion mixture (Figure 3). X-ray diffraction intensities are as graded in Table I.

X-ray diffraction analysis (Figure 3, no. 9). The supernatant of this fraction did not yield a precipitate upon the addition of alcohol. When, however, albumin was first added to the supernatant of a similar control preparation, a precipitate readily formed upon the addition of ethanol to 80 per cent vol/vol. This precipitate did *not* show a crystalline pattern, but merely displayed the expected, diffuse protein scattering.

Samples of reprecipitated gelatin formed from the collagen of aorta at autoclave temperature (Fraction C) were also subject to X-ray diffraction analysis, as were samples of acid mucopolysaccharide-protein complexes (Fraction A) which were extracted by the initial homogenization in cold water. No trace of a crystalline pattern was obtained from these fractions, consequently any close structural bond (not disrupted by the isolation procedure) between hydroxyapatite and materials in these fractions appeared to have been excluded.

DISCUSSION

These studies confirm the presence of a hydroxyapatite as the principal crystallite of human aorta. In addition, they provide evidence that this mineral may be closely associated with isolated elastin, rather than with collagen, polysaccharide or lipid. The AER, isolated by the procedure detailed above, showed an increase of calcium and a decrease of nitrogen (expressed as per cent) with increasing age. These findings are in agreement with those of Lansing, Alex and Rosenthal (3), although the yields and percentages of calcium differ from those reported by them. This difference is undoubtedly due to the inclusion in our specimens of intimal calcific deposits. X-ray diffraction analysis of AER has shown a decrease of protein content, coincident with an increase of crystalline hydroxyapatite with advancing age. These changes are probably related to the chemical modifications described above.

The intimate structural association of hydroxyapatite with elastin has been established by the following evidence.

1. Isolated AER gave the characteristic X-ray diffraction pattern of hydroxyapatite, while neither collagen prepared as gelatin, nor water-soluble polysaccharide fractions yielded crystalline patterns.

2. Elastase treatment of AER liberated into the supernatant solution of the digestion mixtures a nondialyzable protein, which, upon ethanol precipitation, showed hydroxyapatite structure on X-ray diffraction analysis.

3. Powder diagrams of samples not rotated in the camera served to exclude the possibility that the crystallites floating in the supernatant solution were separated from those in the precipitate on the basis of size alone. Since insoluble hydroxyapatite could not be found in the aqueous supernatant of suspensions of elastin *not* treated with elastase, intimate bonds between elastin and these small hydroxyapatite crystallites were presumed to exist. Probably these are strong molecular forces between protein and crystal, since they survive autoclave conditions.

4. The coincidental precipitation of small hydroxyapatite crystals floating in suspensions of

dialyzed elastin by another soluble protein such as albumin has not been observed.

Postulation of elastin-mineral association would not exclude a much weaker, yet physiologically significant association of hydroxyapatite with collagen or lipid which would be disrupted by an extraction procedure involving fat solvents and autoclave temperatures. Indeed, were extraction to break such forces, insoluble hydroxyapatite crystallites would be found in the elastin fractions as finally isolated.

Increased crystallinity of aortic elastin with aging may be sufficient to account for the embrittlement thought to be responsible for hemodynamic changes in the aging aorta, whose elastic properties have been attributed to macromolecular aggregation (18). Crystallization in polymeric substances usually diminishes their elastic properties.[4] Examples of this phenomenon are found in natural and synthetic rubbers, as well as in synthetic fibers. Crystallization of such polymers was found to be associated with an increase of stiffness. A tenfold increase in Young's modulus during crystallization was shown in results obtained on unvulcanized rubber as well as on Neoprene (19). Impregnation of fibrous proteins by many small crystallites would thus increase brittleness by the formation of anchor-points for elastin fibers, with a concomitant decrease of their rubber-like mobility. Amorphous deposits do not show these effects, whereas crystallites by means of secondary valence forces (e.g., hydrogen bonds), can establish firm linkages with fibers and thus establish anchor-points (19). In view of these functional considerations, studies of crystallinity may be more fruitful in the study of aging tissues than simple chemical analysis which does not distinguish between crystalline and amorphous deposits of mineral.

Further quantitative studies of the crystal-fiber relationship in blood vessel wall seem appropriate. Since the number and size of crystallites are of vital importance in determining strength and elastic properties of analogous polymer systems, techniques such as low-angle X-ray scattering and Fourier analysis of line profiles (20) should give valuable quantitative information in this regard. Such studies, using the elastin preparations described above, are now in progress.

[4] While this study has been concerned with the deposition and/or formation of mineral crystal upon a polymer, polymers themselves may exhibit crystallinity under appropriate conditions. The resultant loss of elastic properties is essentially similar.

SUMMARY

1. Elastin residues from human aortas have been prepared on the basis of their insolubility in water at autoclave temperature.

2. The crystallites associated with such residues became prominent in aortas from older individuals and were identified as hydroxyapatite by X-ray diffraction analysis.

3. In aortas obtained from young subjects, only a diffuse X-ray scattering characteristic of protein was observed; this scattering decreased with advancing age.

4. These X-ray findings paralleled a decrease in nitrogen and an increase in the calcium content of isolated aortic elastin residues with advancing age.

5. The intimate structural association of hydroxyapatite crystallites with elastin in aortic tissue has been demonstrated by means of elastase. Treatment of elastin residues with this enzyme caused the liberation of a protein into the nondialyzable supernatant solutions of the digestion mixture, which upon ethanol precipitation gave the typical diffraction pattern of hydroxyapatite.

6. Neither collagen nor polysaccharide fractions obtained from human aortas exhibited crystallite patterns.

ACKNOWLEDGMENT

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REFERENCES

1. Duff, G. L., and McMillan, G. C. Pathology of atherosclerosis. Amer. J. Med. 1951, **11**, 92.
2. Lansing, A. I. Chemical Morphology of Elastic Fibers *in* Trans., Second Josiah Macy Jr. Foundation Conference on Connective Tissues. New York, 1951, p. 45 ff.
3. Lansing, A. I., Alex, M., and Rosenthal, T. B. Calcium and elastin in human arteriosclerosis. J. Geront. 1950, **5**, 112.

4. Carlström, D., Engfeldt, B., Engström, A., and Ringertz, N. Studies on the chemical composition of normal and abnormal blood vessel walls. 1. Chemical nature of vascular calcified deposits. Lab. Invest. 1952, **2**, 325.
5. Carlström, D. X-ray crystallographic studies on apatites and calcified structures. Acta radiol. (Stockh.) suppl. 121, 1955.
6. Lowry, O. H., Gilligan, D. R., and Katersky, E. M. The determination of collagen and elastin in tissues, with results obtained in various normal tissues from different species. J. biol. Chem. 1941, **139**, 795.
7. Ayer, J. P., Hass, G. M., and Philpott, D. E. Aortic elastic tissue; isolation with use of formic acid and discussion of some of its properties. A.M.A. Arch. Path. 1958, **65**, 519.
8. Partridge, S. M., Davis, H. F., and Adair, G. S. The chemistry of connective tissue. 2. Soluble proteins derived from partial hydrolysis of elastin. Biochem. J. 1955, **61**, 11.
9. Gross, J. The structure of elastic tissue as studied with the electron microscope. J. exp. Med. 1949, **89**, 699.
10. Balo, J., and Banga, I. The elastolytic activity of pancreatic extracts. Biochem. J. 1950, **46**, 384.
11. Kirk, J. E., and Dyrbye, M. Hexosamine and acid-hydrolyzable sulfate concentrations of the aorta and pulmonary artery in individuals of various ages. J. Geront. 1956, **11**, 273.
12. Malawista, I., and Schubert, M. Chondromucoprotein; new extraction method and alkaline degradation. J. biol. Chem. 1958, **230**, 535.
13. Lazzarini, A. A., and Weissmann, G. The effect of polyvalent cobalt salts upon human cells in tissue culture. Science 1960, **131**, 1736.
14. Adair, G. S., Davis, H. F., and Partridge, S. M. A soluble protein derived from elastin. Nature (Lond.) 1951, **167**, 605.
15. Schloss, B. The colorimetric determination of glucosamine. Analyt. Chem. 1951, **23**, 1321.
16. Troll, W., and Canman, R. K. A modified photometric ninhydrin method for the analysis of amino and imino acids. J. biol. Chem. 1953, **200**, 803.
17. Posner, A. S., and Stephenson, S. R. Isomorphous substitution in enamel apatite. J. Amer. dent. Ass. 1953, **46**, 257.
18. King, A. L. Circulatory system: Arterial pulse; wave velocity *in* Medical Physics, O. Glasser, Ed. Chicago, Year Book Publishers, 1950, vol. 2, p. 188 ff.
19. Wood, L. A. Crystallization phenomena in natural and synthetic rubbers. Advanc. Colloid Sci. 1946, **2**, 71.
20. Warren, B. E., and Averbach, B. L. The separation of cold-work distortion and particle-size broadening in x-ray patterns. J. appl. Phys. 1952, **23**, 497.

$Co^{58}B_{12}$ ABSORPTION, PLASMA TRANSPORT AND EXCRETION IN PATIENTS WITH MYELOPROLIFERATIVE DISORDERS, SOLID TUMORS AND NON-NEOPLASTIC DISEASES *

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Several investigators have documented the fact that chronic myelocytic leukemia (CML) is associated with increases in serum vitamin B_{12} concentrations which may be of the order of 20 to 40 times the normal value (1–4). Less striking and less consistent elevations have been found in other myeloproliferative disorders. These include acute myelocytic leukemia (1, 3, 4), myeloid metaplasia (2–4), polycythemia vera associated with myelocytic leukemia (4) and Di Guglielmo's disease (5). Elevations have also been reported in patients with solid tumors when there are metastases to the liver (3, 6). The present study was designed to investigate *in vivo* the role that gastrointestinal absorption, plasma transport and renal excretion of vitamin B_{12} play in producing this abnormality.

Previous *in vivo* studies have dealt with the fate of a large intravenously administered dose of radioactive B_{12} (7, 8). In the present studies a dose of approximately 0.5 μg of $Co^{58}B_{12}$ was chosen, since this is within the limits of the estimated daily requirement of B_{12} (9). Oral administration was used so that gastrointestinal absorption could be evaluated and a physiologic pattern of plasma concentrations and excretion could be obtained. Twenty-four subjects have been studied, six of whom had CML.

MATERIALS AND METHODS

Materials. $Co^{58}B_{12}$ [1] had an original specific activity of 3.0 μc per μg. The biologic activity of the material used was confirmed by microbiologic assay (*Lactobacillus leichmannii*) and found to agree within 93 per cent of that given by the manufacturer.

Dosage and administration. The dose administered ranged between 0.33 and 0.56 μg of $Co^{58}B_{12}$. One subject, J.G., received 2.7 μg. Because of the relatively short 72 day half-life of Co^{58}, the calculated activity of the administered dose ranged between 0.3 and 1.4 μc, depending on the time of its administration. After an overnight fast, the subject drank the labeled vitamin along with 50 ml of distilled water. Breakfast was deferred for 1 hour.

Plasma concentrations. Ten ml of heparinized blood was drawn from an antecubital vein at 0, 3, 4.5, 6, 9, 12, 24, 36, 48 and 72 hours and then daily for at least 10 days. In a few studies, bloods were drawn at intervals up to 8 weeks. Plasma was separated by centrifugation and transferred to a 4 ml counting vial for radioassay.

Fecal excretion. Individual stools were collected in metal containers and were assayed for gross activity over the open well of a scintillation counter to evaluate the day-to-day pattern of excretion. Stools were then combined into 4-day pools and homogenized. A 100 g aliquot of each homogenized pool was placed in a 500 ml bottle and counted in the open well of a scintillation counter. A standard was diluted to 100 ml and counted in the same manner. The total fecal excretion over a 12 to 16 day period following administration of $Co^{58}B_{12}$ was determined and expressed as percentage of dose administered. This value subtracted from 100 per cent gave the percentage of dose absorbed.

Urine excretion. Twenty-four-hour urines were collected for 14 days, 300 ml aliquots of each 24-hour specimen were counted in a manner similar to that used for fecal homogenates.

Radioassay. Plasma, feces and urine were counted in a thallium-activated sodium iodide well-type scintillation counter.[2] Plasma samples were counted for a sufficient period of time to give a counting error of less than 5 per cent. Plasma activity was at a low level but at the time of peak activity was always 8 to 30 SD above background. The average background during the course of these studies was 183 cpm. Twelve cpm was equal to twice the SD of the average background. A suitably diluted 4 ml standard of $Co^{58}B_{12}$ was counted each day, and

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[1] Obtained from Merck and Co., Rahway, N. J.

[2] Nuclear Chicago scaler, model number D181, and radiation analyzer, model number 1810.

the radioactivity of each plasma sample was converted to $\mu\mu$g of $Co^{58}B_{12}$ per ml plasma. Fecal specimens were counted with an error of less than 3 per cent. Urine specimens were counted for 20 minutes to demonstrate negligible radioactivity.

Microbiologic assay. Blood specimens from fasting patients were drawn on the morning of the study prior to administration of the tracer dose and assayed in quadruplicate for their B_{12} concentration by a modification, described in detail elsewhere (3), of the USP method using *L. leichmannii*, A.T.C.C. 7830. With this method the mean serum B_{12} concentration for 31 normal subjects was 533 $\mu\mu$g per ml of serum with a range of 260 to 850.

Subjects. The 24 patients chosen for this study, along with pertinent clinical data, are listed in Table I. All diagnoses were established both clinically and histologically. None of the patients studied had received oral or parenteral vitamins for at least 1 month prior to the study. All but 3 patients were studied during residency on the metabolism unit of the National Cancer Institute where they were fed diets of known composition. Their stool and urine collections were rigidly controlled according to previously described procedures (10). Each patient was ambulatory during the study.

Subjects have been classified according to the following diagnoses: non-neoplastic diseases, solid tumors, myeloid metaplasia, chronic myelocytic leukemia, hypersplenism and multiple myeloma. For the most part, subjects with non-neoplastic diseases and solid tumors displayed findings similar to those noted by other investigators in normal subjects given a 0.5 μg oral dose of $Co^{58}B_{12}$ and therefore in the discussion are referred to as control subjects.

RESULTS

Serum B_{12} concentrations (Table I)

Four of the five patients with non-neoplastic disease had serum B_{12} concentrations within 2 SD of the normal mean. The fifth patient, whose diagnosis was primary pulmonary hypertension had a moderately increased serum B_{12} concentration of 1,213 $\mu\mu$g per ml.

The five patients with solid neoplasms had a mean serum B_{12} concentration of 577 $\mu\mu$g per ml and a range of 330 to 890 $\mu\mu$g per ml. Two of the four patients with myeloid metaplasia had moderately increased serum B_{12} concentrations. The other two had normal concentrations. The mean concentration for this group was 1,129 $\mu\mu$g per ml with a range of 420 to 2,100.

The seven patients with CML (including one diagnosed as eosinophilic leukemia) demonstrated striking increases in serum B_{12} concentration, the mean concentration being 8,550 $\mu\mu$g per ml with a range of 1,800 to 17,700 $\mu\mu$g per ml. Patient D.L. was in clinical and hematological remission and Patients J.L. and P.P. were in partial relapse at the time they were studied. The two patients with hypersplenism and pancytopenia had serum B_{12} concentrations at the lower limits of normal, 195 and 200 $\mu\mu$g per ml. The one subject with multiple myeloma had a normal serum B_{12} concentration of 625 $\mu\mu$g per ml.

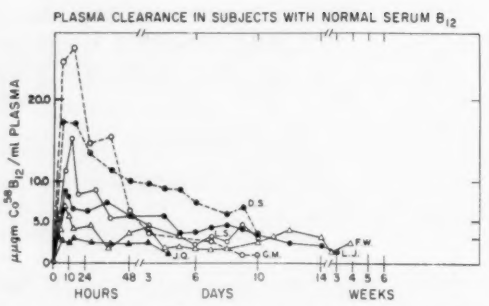

FIG. 1. PLASMA $Co^{58}B_{12}$ CONCENTRATIONS FOLLOWING ORAL ADMINISTRATION TO SUBJECTS WITH A NORMAL SERUM B_{12}. J.Q., L.S., G.M. and F.W., non-neoplastic diseases. D.S. and L.J., embryonal sarcoma.

Gastrointestinal absorption

Since all of the patients but J.G. received a dose of $Co^{58}B_{12}$ in the 0.5 μg range, the percentage of oral dose absorbed has been used as a basis for comparisons between patients. The percentage absorbed by each of the 24 subjects is listed in Table I. The mean for all of the 24 subjects was 66.8 per cent with a range of 30 to 81 per cent. No significant difference between the various disease groups studied was noted in terms of the percentage of dose absorbed. No correlation between a patient's serum B_{12} concentration and the percentage of dose absorbed could be made.

J.G., who received a dose of 2.73 μg, had an absorption of 71 per cent of the administered dose, a value not significantly different from that noted in the others.

Plasma concentrations of $Co^{58}B_{12}$

Curves of plasma radioactivity were obtained in 19 of the 24 patients. For the purpose of this study, the rate of plasma disappearance has been evaluated by noting the $T_{\frac{1}{2}}$.[3]

[3] Since plasma curves had both an appearance phase as well as a disappearance phase, and since the disappearance phase was not exponential, the use of this term

TABLE I

Clinical data on 24 subjects given an oral dose of $Co^{58}B_{12}$; data obtained on gastrointestinal absorption and plasma concentration

Subject	Sex	Age	Diagnosis	Clinical status		Serum B_{12}	Dose of $Co^{58}B_{12}$	Absorbed	Plasma $Co^{58}B_{12}$ Peak concentration	Plasma $Co^{58}B_{12}$ Time of peak	"$T_{1/2}$"*
					WBC/mm³	*μμg/ml*	*μg*	*%*	*μμg/ml*	*hrs*	*days*
J.Q.	F	33	Partial resection of ileum	Stable	6,000	800	0.37	60	3	12	4
L.S.	M	22	Primary pulmonary hypertension	Stable	7,500	1,213	0.49	86	15	10	1.2
S.M.	F	32	Rheumatic heart disease	Stable	10,200	550	0.49	69			
F.W.	F	68	Subtotal gastrectomy	Weight loss	6,000	465	0.49	76	7	8	4
G.M.	F	42	Anorexia nervosa	Weight loss	8,200	790	0.56	76	27	12	2
L.J.	F	16	Embryonal sarcoma	Stable	7,500	725	0.49	80	9	7	5
R.L.	M	20	Hepatoma	Partially resected	9,000	462	0.49	62	13	35	>9
H.C.	M	52	Prostatic carcinoma	Bone metastases	3,000	330	0.48	75			
D.S.	M	15	Embryonal sarcoma	Metastases	5,400	478	0.56	41	18	5	7
W.P.	M	54	Pancreatic carcinoma	Untreated	7,600	890	0.56	68			
W.J.	M	61	Myeloid metaplasia	Untreated	4,000	2,100	0.33	80	15	48	9
S.P.	M	65	Myeloid metaplasia	Untreated	8,000	1,215	0.48	30	28	10d	?
J.G.	M	50	Myeloid metaplasia	Untreated	9,000	780	2.73	71	16	9	?
M.G.	F	64	Myeloid metaplasia	Untreated	3,000	420	0.48	52	20	6d	?
D.L.	M	8	Chronic myelocytic leukemia	Remission	8,000	17,700	0.48	81	71	48	13
J.L.	F	23	Chronic myelocytic leukemia	Partial relapse	5,600	8,700	0.49	76	54	72	4
O.F.	M	48	Chronic myelocytic leukemia	Untreated	200,000	6,800	0.48	55	8	72	8
P.P.	M	36	Chronic myelocytic leukemia	Partial relapse	40,000	6,700	0.48	78	18	24	11
M.B.	F	37	Chronic myelocytic leukemia	Untreated	194,000	6,150	0.48	48	30	36	13
R.F.	M	52	Chronic myelocytic leukemia	Untreated	190,000	1,800	0.48	78			
L.E.	M	25	Eosinophilic leukemia	Untreated	17,000	12,000	0.49	79	22	36	15
C.F.	M	40	Hypersplenism with pancytopenia	Untreated	2,500	195	0.48	81	14	72	?
P.K.	M	55	Hypersplenism with pancytopenia	Untreated	3,000	200	0.49	50	8	6	0.5
J.M.	F	60	Multiple myeloma	Untreated	6,000	625	0.48	51			

* See Footnote 3 in text.

Non-neoplastic disease and solid tumor groups. A grossly similar pattern of plasma radioactivity was noted in four patients with non-neoplastic diseases and in two patients with solid tumors (Figure 1). Significant plasma radioactivity was noted within the first 3 to 4.5 hours after administration of $Co^{58}B_{12}$. Thereafter, plasma radioactivity increased in an approximately linear manner until a peak level was attained at a mean of 9 hours (range, 5 to 12 hours). The peak radioactivity

in the present context is somewhat unconventional. In the present paper "$T_{\frac{1}{2}}$" has been arbitrarily defined as the time interval between peak plasma radioactivity and the time when plasma concentration of $Co^{58}B_{12}$ falls to half of the peak concentration.

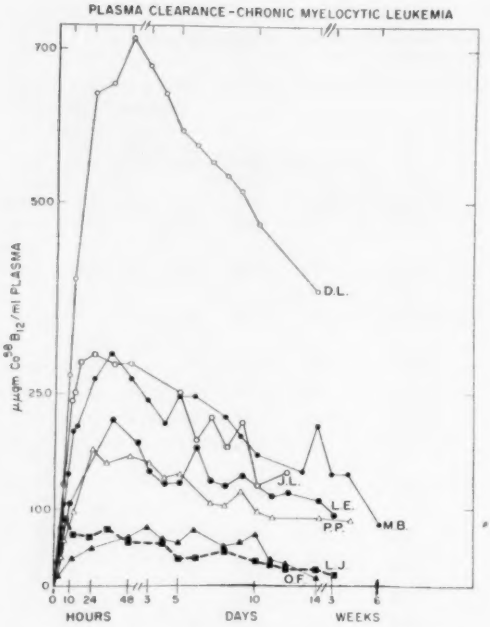

FIG. 2. PLASMA $Co^{58}B_{12}$ CONCENTRATIONS FOLLOWING ORAL ADMINISTRATION OF 0.5 μG TO SUBJECTS WITH CHRONIC MYELOCYTIC LEUKEMIA. L.E., eosinophilic leukemia. L.J., embryonal sarcoma, a characteristic pattern of subjects with a normal serum B_{12} included for comparison.

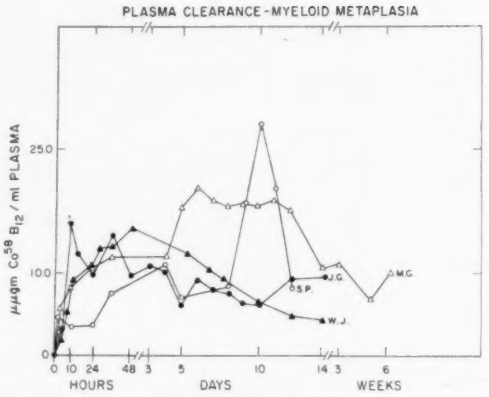

FIG. 3. PLASMA $Co^{58}B_{12}$ CONCENTRATIONS FOLLOWING ORAL ADMINISTRATION OF 0.5 μG TO FOUR SUBJECTS WITH MYELOID METAPLASIA.

was equivalent to a mean of 13 $\mu\mu$g of $Co^{58}B_{12}$ per ml of plasma (range, 3 to 27 $\mu\mu$g per ml). Subsequently, there was a gradual and quite irregular decline in plasma radioactivity during the next 10 to 14 days. The $T_{\frac{1}{2}}$ of plasma disappearance had a mean value of 4 days (range, 1.5 to 7 days). Three of these six patients had significant radioactivity in their plasma for as long as 10 days after administration of the $Co^{58}B_{12}$.

A seventh patient, R.L., whose diagnosis was hepatoma, had a peak concentration of 13 $\mu\mu$g $Co^{58}B_{12}$ per ml of plasma. The peak, however, occurred at 35 hours. The plasma disappearance was also delayed with a $T_{\frac{1}{2}}$ of > 9 days.

Chronic myelocytic leukemia. Figure 2 summarizes the plasma $Co^{58}B_{12}$ concentrations observed in seven patients with chronic myelocytic leukemia. (Subject L.E. with eosinophilic leukemia is included in this group because of his similar pattern.) The initial portion of their plasma *appearance* curves was approximately linear and had a slope similar to that of the previous group. At from 10 to 14 hours, however, instead of reaching a peak concentration, serial plasma samples continued to rise more slowly, resulting in a delay in reaching peak concentrations. The latter did not occur until a mean of 2 days (range, 1 to 3 days). In general, plasma radioactivity also reached much higher concentrations, being equivalent to a mean of 34 $\mu\mu$g $Co^{58}B_{12}$ per ml of plasma (range, 8 to 71 $\mu\mu$g per ml). In addition, there was delayed plasma disappearance with a mean $T_{\frac{1}{2}}$ of 10.7 days (range, 4 to 15 days). At the end of 10 days, four of the six patients retained in their plasma 56 to 88 per cent of their peak plasma concentration. A plasma sample drawn on M.B. at 6 weeks demonstrated significant residual radioactivity.

Myeloid metaplasia. Inspection of the plasma radioactivity curves for the four patients with myeloid metaplasia (Figure 3) reveals that, except for one subject, W.J., whose curve has the features noted for patients with chronic myelocytic leukemia, these patients had bizarre and irregular *appearance* and *disappearance* curves. They had a mean peak plasma concentration of 20 $\mu\mu$g $Co^{58}B_{12}$ per ml of plasma (range, 15 to 28 $\mu\mu$g per ml). Because of the very irregular plasma disappearance, $T_{\frac{1}{2}}$ could not be evaluated. In general, however, these patients demonstrated more residual $Co^{58}B_{12}$ in their plasma at the end of 10 days than did the non-neoplastic disease-solid tumor group.

Others. P.K., a patient with hypersplenism and pancytopenia, had a plasma $Co^{58}B_{12}$ curve similar to the non-neoplastic disease and solid tumor groups. C.F., on the other hand, with hypersplenism and pancytopenia, had a delayed plasma peak occurring at 3 days. Plasma activity was not studied in the single case of multiple myeloma.

Urinary excretion of $Co^{58}B_{12}$

There was no detectable urinary excretion of radioactive material in the unconcentrated urine specimens of any of the subjects studied. Calculations indicate that the method used in the present study would have detected excretion of more than 1 per cent of the absorbed dose per 24 hour urine specimen.

DISCUSSION

The studies indicate that gastrointestinal absorption of B_{12}, as measured by the fecal excretion technique, is not increased in the patient with CML. The percentage of administered $Co^{58}B_{12}$ absorbed had an over-all mean of 66.8, with a range of 30 to 81 for the 24 subjects studied. These figures are in good agreement with those reported by Halsted and colleagues (11) who found that with a 0.5 μg dose of radioactive B_{12}, normal subjects absorbed 43 to 95 per cent of the dose, with a mean of 66 and a maximum variation within the same patient of 18 per cent. The absorption in seven subjects with CML in the present study did not differ from this range, nor was the amount absorbed abnormal in the two subjects with myeloid metaplasia and a high serum B_{12}. Heinrich and Erdmann-Oehlecker (12) have also found normal gastrointestinal absorption of B_{12} in one patient with CML given 1 μg of B_{12}.

A corollary to these findings is that the serum concentration of B_{12} per se does not control the amount of B_{12} absorbed. Both Patient L.J., with a serum B_{12} of 725 $\mu\mu$g per ml and Patient D.L., with a concentration of 17,700 $\mu\mu$g per ml, absorbed approximately 80 per cent of the test dose. The data also indicate that the plasma concentration of "unsaturated B_{12}-binding protein" does not greatly influence the amount of B_{12} absorbed, since plasma from patients with CML has a markedly increased unsaturated B_{12}-binding capacity (13–16).

It would appear, therefore, that the high plasma B_{12} concentrations seen in CML are not due to excessive gastrointestinal absorption of the vitamin. In addition, it is unlikely that the abnormality is caused by impaired renal excretion, since the total daily urinary excretion of vitamin B_{12} is normally very small, viz., about 0.1 μg per day (17), and $Co^{58}B_{12}$ was undetectable in the urine of both control and CML patients in the present study.

We believe that the high B_{12} content of CML plasma is due to a relative shift of B_{12} from tissue sites to plasma. It can be calculated from known data (18) that the transfer of only 3 per cent of the normal liver's content of B_{12} to the plasma compartment would be sufficient to produce the tenfold increase in plasma B_{12} seen in CML. This possibility would, of course, be excluded should studies reveal that CML is associated with a high tissue content as well as high plasma concentration of the vitamin. Data on tissue concentrations of B_{12} in CML are limited. In the single autopsied case studied by Mollin and Ross (2) the total B_{12} content of liver, spleen and muscle was within the normal range. Nelson has studied the B_{12} content of liver tissue obtained by needle biopsy in two cases of myelocytic leukemia associated with a high serum B_{12} and found it to be approximately 30 per cent below that of the average normal control (19).

The marked differences between control and CML subjects in plasma radioactivity curves following oral administration of $Co^{58}B_{12}$ provide evidence for a disturbed kinetics of B_{12} in CML. An analysis of these curves necessitates a consideration of the factor involved in the absorption, plasma transport and distribution of B_{12}. These are summarized in schematic form in Figure 4. Because of gaps in our present knowledge the scheme is tentative.

The delay in peak plasma concentration (9 hours in control patients) following an oral dose of $Co^{58}B_{12}$ has been noted by other investigators (20, 21) and appears to be due to a delay in the movement of B_{12} across the small bowel mucosa (21–23). The initial slope of the plasma *appearance* curve for the CML patients appears to be comparable with that of the control patients (Figure 2) suggesting a normal rate of entry into the

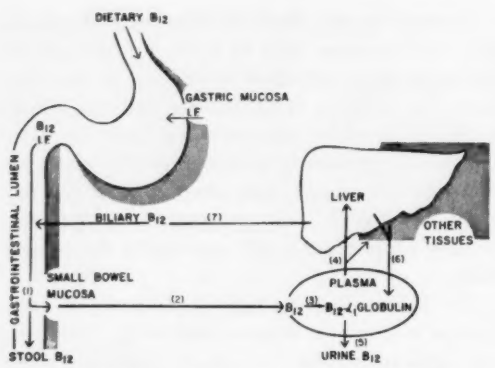

FIG. 4. A SCHEMATIC MODEL OF VITAMIN B_{12} METABOLISM. Reaction rates, (1 through 7). I.F. is intrinsic factor. It is postulated that in chronic myelocytic leukemia there is an increased plasma B_{12} concentration because of a relative shift of tissue stores of B_{12} to the plasma via 6 as well as increased retention of exogenous B_{12} within the plasma compartment via 3. References and further details are given in the Discussion.

plasma compartment. A greater than normal delay in peak plasma concentration (24 hours) was noted in the CML patients and is discussed below.

In both the control and CML subjects the plasma *disappearance* curves had an irregular downward slope. We believe that this is due to a complex recycling of B_{12} through the plasma compartment. This is supported by the accumulating evidence for an enterohepatic circulation of B_{12} (24, 25) as well as by the studies of Miller, Corbus and Sullivan (8) and Glass and Schaffer (22) which suggest that there is a transfer of absorbed B_{12} from plasma to unidentified compartment(s) prior to its eventual deposition in the liver.

Because of the complex nature of the plasma radioactivity curves, in the present study $T_{\frac{1}{2}}$ is defined as the time following peak radioactivity when plasma radioactivity falls to and remains below half the peak concentration. The mean value for control and CML patients was 4 and 11 days, respectively. Employing an intravenous dose of 1.5 or 4 μg of radioactive B_{12} Mollin, Pitney, Baker and Bradley (7) and Miller and colleagues (8) have demonstrated that in patients with CML there is a diminished plasma disappearance rate for injected B_{12}. The rates reported were much more rapid than those obtained in the present study and are probably due to the unphysiologic effects of administering a large dose of free B_{12} intravenously. This is especially true in normal subjects who have a relatively limited plasma unsaturated B_{12}-binding capacity (see below) and, therefore, might be expected to have a rapid loss of free B_{12} from the plasma.

The slower plasma disappearance rate combined with the prolonged influx at a normal rate into the plasma from the small bowel of the tracer dose of $Co^{58}B_{12}$ could, by a cumulative effect, account for the higher than normal but more delayed peak plasma concentration of $Co^{58}B_{12}$ seen in the patients with CML in the present study. In patients with CML, a given dose of $Co^{58}B_{12}$ enters a plasma B_{12} pool which is approximately 10 times normal size. Therefore, the delayed plasma disappearance of the labeled vitamin may merely represent an isotope dilution effect. Since CML plasma has a high unsaturated B_{12}-binding capacity (13–16), an additional factor may be that as the tracer dose enters the plasma compartment a greater than normal fraction of it is tightly bound to plasma protein.

In both normal subjects and patients with CML, 80 to 90 per cent of total plasma B_{12} is tightly bound to an α_1-globulin present in the seromucoid fraction of plasma (16). Since, within a few hours following an oral dose of $Co^{58}B_{12}$, the radioactive B_{12} appearing in plasma is also in this form (13) it seems reasonable to assume that, once absorbed, an orally administered dose of $Co^{58}B_{12}$ rapidly equilibrates with the total plasma B_{12}. Using the previously mentioned disappearance rates it seems possible, therefore, to calculate the daily plasma turnover of vitamin B_{12}. For a control subject with a plasma B_{12} of 0.5 mμg per ml, a plasma volume of 2,500 ml and a $T_{\frac{1}{2}}$ of four days, the plasma turnover would be 0.16 μg per day. Similar calculations for a patient with CML, assuming a plasma B_{12} of 5 mμg per ml, a plasma volume of 2,500 ml and a $T_{\frac{1}{2}}$ of 11 days, give a plasma turnover of 0.57 μg per day.

If plasma turnover of B_{12} is indeed increased in CML, the abnormality in B_{12} metabolism may be more fundamental than merely a shift of tissue stores of B_{12} to the plasma compartment resulting from an excess of plasma B_{12}-binding protein. Clearly, the assumptions underlying the calculations and the validity of the disappearance rates obtained bear further investigation.

SUMMARY

1. Gastrointestinal absorption, plasma transport and urinary excretion of radioactivity following oral administration of 0.5 μg of $Co^{58}B_{12}$ have been evaluated in 24 patients, 7 of whom had chronic myelocytic leukemia.

2. The amount absorbed by all subjects, as measured by stool excretion, had a mean of 67 per cent. There was no correlation between diagnosis or serum B_{12} concentration and percentage of dose absorbed.

3. Subjects with non-neoplastic diseases as well as patients with solid tumors, all of whom had a normal serum B_{12} concentration, had a similar pattern of plasma clearance. $Co^{58}B_{12}$ reached a mean peak concentration of 13 $\mu\mu$g per ml of plasma at 9 hours and fell to half the peak concentration at a mean of 4 days. In seven patients with chronic myelocytic leukemia, $Co^{58}B_{12}$ reached a mean peak plasma concentration of 33 $\mu\mu$g per ml at 2 days and fell to half the peak concentration at a mean of 11 days.

4. No detectable urinary excretion of radioactive material in unconcentrated urine specimens was noted in any of the subjects studied.

5. The increased serum concentration of vitamin B_{12} seen in patients with chronic myelocytic leukemia does not appear to be due to increased gastrointestinal absorption or decreased urinary excretion of the vitamin. A relative shift of B_{12} from tissue sites to plasma appears to be the most likely mechanism.

6. Calculations based upon the plasma disappearance rates obtained in the present study suggest that, in addition to the increased concentration of B_{12} and B_{12}-binding protein found in plasma of patients with chronic myelocytic leukemia, there is an increased plasma turnover of the vitamin in this disease.

REFERENCES

1. Beard, M. F., Pitney, W. R., and Sanneman, E. H., Jr. Serum concentrations of vitamin B_{12} in patients suffering from leukemia. Blood 1954, **9**, 789.
2. Mollin, D. L., and Ross, G. I. M. Serum vitamin B_{12} concentrations in leukaemia and in some other haematological conditions. Brit. J. Haemat. 1955, **1**, 155.
3. Mendelsohn, R. S., and Watkin, D. M. Serum vitamin B_{12} concentrations determined by L. leichmannii assay in patients with neoplastic disease. J. Lab. clin. Med. 1958, **51**, 860.
4. Rachmilewitz, M., Izak, G., Hochman, A., Aronovitch, J., and Grossowicz, N. Serum vitamin B_{12} in leukemias and malignant lymphomas. Blood 1957, **12**, 804.
5. Damashek, W. Pernicious anemia, megaloblastosis and the Di Guglielmo syndrome. Blood 1958, **13**, 1085.
6. Grossowicz, N., Hochman, A., Aronovitch, J., Izak, G., and Rachmilewitz, M. Malignant growth in the liver and serum-vitamin-B_{12} levels. Lancet 1957, **1**, 1116.
7. Mollin, D. L., Pitney, W. R., Baker, S. J., and Bradley, J. E. The plasma clearance and urinary excretion of parenterally administered $Co^{58}B_{12}$. Blood 1956, **11**, 31.
8. Miller, A., Corbus, H. F., and Sullivan, J. F. The plasma disappearance, excretion, and tissue distribution of cobalt^{60} labelled vitamin B_{12} in normal subjects and patients with chronic myelogenous leukemia. J. clin. Invest. 1957, **36**, 18.
9. Schilling, R. F. The absorption and utilization of vitamin B_{12}. Amer. J. clin. Nutr. 1955, **3**, 45.
10. Watkin, D. M., and Silver, R. T. Nitrogen, mineral, uric acid and basal metabolism studies in a case of adult acute leukemia with extensive osteolytic bone disease. Amer. J. Med. 1958, **24**, 638.
11. Halsted, J. A., Lewis, P. M., Hvolboll, E. E., Gasster, M., and Swendseid, M. E. An evaluation of the fecal recovery method for determining intestinal absorption of cobalt^{60}-labeled vitamin B_{12}. J. Lab. clin. Med. 1956, **48**, 92.
12. Heinrich, H. C., and Erdmann-Oehlecker, S. Der vitamin B_{12}-Stoffwechsel bei Hämoblastosen. III. Resorption, Blutverteilung, Serumproteinbindung, Retention und Exkretion der B_{12}-Vitamine bei Hämoblastosen nach oraler und parenteraler B_{12}-Applikation. Clin. chim. Acta 1956, **1**, 326.
13. Pitney, W. R., Beard, M. F., and Van Loon, E. J. Observations on the bound form of vitamin B_{12} in human serum. J. biol. Chem 1954, **207**, 143.
14. Meyer, L. M., Bertcher, R. W., and Cronkite, E. P. Serum Co^{60} vitamin B_{12} binding capacity in some hematologic disorders. Proc. Soc. exp. Biol. (N. Y.) 1957, **96**, 360.
15. Miller, A. The *in vitro* binding of cobalt^{60} labelled vitamin B_{12} by normal and leukemic sera. J. clin. Invest. 1958, **37**, 556.
16. Weinstein, I. B., Weissman, S. M., and Watkin, D. M. The plasma vitamin B_{12} binding substance: I. Its detection in the seromucoid fraction of plasma from normal subjects and patients with chronic myelocytic leukemia. J. clin. Invest. 1959, **38**, 1904.
17. Mollin, D. L., and Ross, G. I. M. The vitamin B_{12} concentrations of serum and urine of normals and of patients with megaloblastic anaemias and other diseases. J. clin. Path. 1952, **5**, 129.

18. Nelson, R. S., and Doctor, V. M. The vitamin B_{12} content of human liver as determined by bio-assay of needle biopsy material. Ann. intern. Med. 1958, **49**, 1361.
19. Nelson, R. S. Personal communication.
20. Booth, C. C., and Mollin, D. L. Plasma, tissue and urinary radioactivity after oral administration of ^{58}Co-labeled vitamin B_{12}. Brit. J. Haemat. 1956, **2**, 223.
21. Doscherholmen, A., and Hagen, P. S. Radioactive vitamin B_{12} absorption studies: Results of direct measurement of radioactivity in the blood. Blood 1957, **12**, 336.
22. Glass, G. B. J., and Schaffer, H. Further observations on the intestinal absorption of vitamin B_{12} as measured by hepatic uptake of $Co^{60}B_{12}$. Bull. N. Y. med. Coll. 1956, **19**, 74.
23. Doscherholmen, A., and Hagen, P. S. Delay of absorption of radiolabeled cyanocobalmin in the intestinal wall in the presence of I. F. J. Lab. clin. Med. 1959, **54**, 434.
24. Grasbeck, R., Nyberg, W., and Reizenstein, P. Biliary and fecal vitamin B_{12} excretion in man; an isotope study. Proc. Soc. exp. Biol. (N. Y.) 1958, **97**, 780.
25. Okuda, K., Grasbeck, R., and Chow, B. F. Bile and vitamin B_{12} absorption. J. Lab. clin. Med. 1958, **51**, 17.

STUDIES OF UNBOUND AMINO ACID DISTRIBUTIONS IN PLASMA, ERYTHROCYTES, LEUKOCYTES AND URINE OF NORMAL HUMAN SUBJECTS

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Understanding of the role of the blood in amino acid transport and metabolism requires information not only about the plasma concentrations and turnover of these substances, but also about their distribution between the plasma and the formed elements of the blood.

Many workers (1–4) have measured the concentrations of unbound amino acids in plasma and several have studied their contents in erythrocytes (5) and leukocytes (6, 7). Simultaneous measurements of unbound amino acids in two or more of the blood compartments are few and limited (8, 9).

The present communication describes methods for the measurement of concurrent concentrations of unbound amino acids in plasma, erythrocytes, leukocytes and urine, and reports the results obtained from the study of normal fasting subjects.

METHODS

Collection of samples. Subjects were instructed to fast from 7 p.m. the evening before until the blood had been drawn. Between 8 and 10 a.m., 50 to 60 ml of blood was collected by the method of Lund, McMenamy and Neville (10) using one of the following resins for decalcification: Rohm and Haas anion exchange resin XE-114, mesh size 20 to 50, one part of which was cycled with ethylenediamine-tetraacetate (EDTA), two parts with chloride and one part uncharged; or, Rohm and Haas anion exchange resin XE-168, mesh size 20 to 50, two parts of which were cycled with EDTA, two parts with chloride and one part uncharged.[1] Decalcified blood as opposed to heparin-treated or citrated blood gave a much better yield of leukocytes. Containers of the blood were immediately placed in ice water for transportation to the laboratory except where otherwise specified. Blood analyses were commenced within one-half hour after the blood collection. Urines were collected during the latter part of the fasting period over a measured time interval of 6 to 8 hours with thymol as preservative.

Preparation of the plasma, erythrocytes and leukocyte samples for analysis. Surfaces that came in contact with blood or blood cells were treated to make them nonwettable (11). Glass was first cleaned with a detergent solution, rinsed thoroughly with water, dried, exposed for several seconds to vapors of Dri-Film 9977 (General Electric Co., Chemical Division), flushed with air, and finally rinsed thoroughly with water. Metal surfaces were cleaned, placed in a boiling solution of 1 per cent Arquad (Armour and Co.) for 2 minutes, then thoroughly rinsed with water.

Solutions used in sample preparation were kept at 2° C unless otherwise stated. For plasma and erythrocyte analyses a 12 ml aliquot of blood was centrifuged in a 15 ml plastic test tube at 1,700 × G for 40 minutes. Four ml of the plasma was removed and dialyzed by the thin layer technique (1), except that here the plasma was placed in Visking tubing, size 22/32, and dialyzed for 2 hours against 25 ml of water. The dialysate was dried from the frozen state in a 25 ml round bottom flask.

The remainder of the contents of the plastic centrifuge tube was frozen, the tube was then cut in two below the residual plasma layer and approximately 4.4 g of frozen red cells was weighed accurately from the plug. The red cells were lysed by thawing, refreezing and rethawing, diluted to a volume of 12 ml with water, placed inside Visking tubing size 23/32, and dialyzed for 2 hours against 40 ml of water. The dialysate of the red cells was agitated for several minutes with 0.5 ml freshly washed EDTA-chloride resin prepared as described under *Collection of samples.* This procedure removes material from the dialysate which causes spreading of the glutamic acid and O-phosphoethanolamine zones. It is filtered and dried from the frozen state in a 50 ml round bottom flask.

To obtain white cells for analysis, 40 to 50 ml of blood from a subject with a normal white count (smaller amounts of blood may be used from patients with elevated counts) was diluted with two parts of a 3 per cent dextran solution at 2° C, gently mixed for 5 minutes

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[1] After autoclaving, the latter resin sheds a small amount of a ninhydrin-positive substance with an Rf value between valine and α-amino-*n*-butyric acid; this substance did not interfere with the analyses.

TABLE I

Concentration of unbound amino acids in plasma

	Standard solution	No. of subjects	Mean
	mmoles/L		*μmoles/kg water*
Alanine	2.14	15	399 ± 25*
α-Amino-*n*-butyric acid	0.32	11	28 ± 3
Arginine	0.53	13	68 ± 6
Glutamic acid	1.94	15	<28
Glutamine	3.75	15	512 ± 24
Histidine	0.74	12	89 ± 9
Lysine	1.88	15	204 ± 13
Methionine	0.32	12	22 ± 2
Ornithine	0.85	11	85 ± 8
Phenylalanine	0.53	14	54 ± 4
Proline	1.07	15	185 ± 15
Threonine	1.39	15	165 ± 6
Tryptophan	0.43	15	(18)†
Tyrosine	0.74	15	70 ± 4
Valine	1.49	15	203 ± 11
Ergothioneine	2.07	15	<10
Ethanolamine	0.74	15	<10
Leucine plus isoleucine	1.60‡	13	202 ± 18
Serine plus glycine	4.27§	14	609 ± 26
Urea	34.4	13	5,382 ± 267

* Standard deviation of the mean.
† Estimated; see text.
‡ Leucine, 0.96 mmole; isoleucine, 0.64 mmole.
§ Serine, 1.5 mmoles; glycine, 2.77 mmoles.

and allowed to stand 20 minutes (12). The dextran solution contained 3 per cent dextran H (R. K. Laros and Co. Bethlehem, Pa.), 0.1 per cent dextrose, and 0.15 M NaCl; it had been filtered through Seitz filters to remove the insoluble particles and decalcified, since calcium leaks from the Seitz filters, with a column of resin (50 ml of the EDTA-chloride resin, described above, to 1,000 of dextran solution).[2] After standing 20 minutes, the supernatant solution was siphoned into a 100 ml pear shaped oil centrifuge tube, the tip of which had been modified by attachment of a Tygon plastic tube (5/16 in. OD, 1/16 in. wall thickness, 2.5 in. long) with the lower end thermally sealed. The solution was centrifuged at 100 × G for 1 hour. The white cell layer was cut from the tube and suspended in 0.5 ml of a 1 per cent acetylated human serum albumin solution in 0.15 M NaCl. The albumin was mildly acetylated using conditions described for Experiment 135 DI, Table I (13), to prevent its binding of tryptophan. The white cell counts and platelet counts were made in quadruplicate on aliquots of the plug suspension. A recovery of 70 to 90 per cent of the white cells was obtained. The plug suspension contained less than 5 per cent of the total blood platelets. Differential counts made on the plug suspension and whole blood did not vary significantly. An aliquot of the plug suspension was centrifuged in a microtube for 20 minutes at 7,800 × G using the centrifuge described by Gold and Solomon (14). The relative volume of packed white cells was taken as the volume fraction of cells in the plug suspension.

The volume of the remainder of the plug suspension, usually 0.6 to 0.8 ml, was measured and made up to 2 ml with water. The cells were lysed by freezing and thawing and dialyzed in Visking tubing size 22/32 for 2 hours against 25 ml of water by the thin layer technique. The dialysate was treated with resin in the same manner as was described for the erythrocyte dialysates and dried from the frozen state in a 25 ml round bottom flask.

The residues of the dialysates from the plasma were dissolved in 0.345 ml of Tergitol-Safranin solution, the composition of which was as follows: 0.05 per cent Tergitol Nonionic NPX (Carbide and Carbon Chemical Co.), 0.005 per cent Safranin O dye (National Aniline Division, Allied Chemical and Dye Corp.) and 0.4 per cent EDTA trisodium salt. The wetting agent in this solution facilitates dissolving the residue in the flask and makes micropipetting more reproducible; the dye serves as a marker to aid in counting the multiple applications of the concentrates placed on the papers; EDTA is added as a general precaution to prevent the interference of bivalent cations in chromatography. The residues of the dialysates of the erythrocytes were dissolved in the Tergitol-Safranin solution on the basis of 0.054 ml per g packed cells dialyzed. The residues of the dialysates of the leukocytes were dissolved in the Tergitol-Safranin solution on the basis of 0.862 ml per ml of packed cells dialyzed. In the calculations, a 14.7 per cent increase in volume of the Tergitol-Safranin solution was allowed for the salts present.

Preparation of the urine samples for analysis. An aliquot of urine equivalent to a 12 minute excretion period was passed at 2° C through a resin column containing 4 ml of wet resin of the same type and prepared in the same manner as the resin used for the collection of blood. The flow rate was adjusted to about 1 ml per minute. The column was washed with a 10 mM solution of NaCl to give a total volume of effluent and wash of 40 ml. The resin step removes substances from the urine which might otherwise precipitate when the urine is concentrated and interfere with chromatography; amino acids were not retained by the resin. A sample of the mixed urine and wash solution was removed for total amino acid estimation (see below). The remainder was lyophilized in a 50 ml round bottom flask.

Concentrations of unbound amino acids in urine vary considerably. In order to estimate the approximate concentration for chromatography, a 50 μl aliquot of the mixed urine and resin wash was placed on Whatman no. 1 paper which had previously been dipped in 0.1 per cent NaOH solution and dried. Fifty μl aliquots of solutions of alanine at concentrations of 0.5, 1, 2 and 4 mM were placed in the vicinity of the samples. The paper was dipped in the ninhydrin reagent, less collidine (1), and placed in an oven for 15 minutes at 60° C for color development. Where the intensity of the zone of the

[2] A dextran (grade H, clinical, lot H 1257) was recently obtained from Laros and Co.; it is free of insoluble particles and can be used without filtering and decalcification. This type was not used in the experiments reported here.

TABLE II

Urinary amino acid excretion rates of normal subjects (μmoles per hour)

	Standard solution	No. of subjects	Fasting urines (paper chromatography)		Nonfasting urines (resin column)*	
			Mean	Range	Mean	Range
	mmoles/L					
Alanine	3.0	15	7.8	3.0–16.0	21.6	9.4–33.0
α-Amino-*n*-butyric acid	0.5	5	<2.0			
Arginine	0.0	15	<1.0		<2.4	
Aspartic acid	0.0	8	<1.0		<3.0	
Glutamic acid	1.0	9	<1.0		<3.0	
Glutamine	4.0	15	17.1	6.0–28.0		
Histidine	8.0	14	32.4†	6.0–80.0	102.0†	41.0–138.0
Lysine	2.0	14	8.4	3.3–22.0	5.4	2.9–14.2
Methionine	0.5	15	<1.0		<3.0	
Ornithine	1.0	15	<2.0			
Phenylalanine	1.0	15	<2.0	<1.5–3.0	4.5	2.5–7.5
Proline	1.0	15	<1.0		<3.5	
Threonine	3.0	14	7.6	4.2–22.0	9.8	5.2–17.5
Tryptophan	0.5	14	<1.7	<0.5–5.0		
Tyrosine	1.0	15	3.1	1.5–6.0	8.0	3.5–11.5
Valine	1.0	15	<2.0		<3.5	
Ethanolamine	2.0	14	14.1	7.8–24.0		
Guanidoacetic acid	2.0	5	9.6	4.0–20.0		
Leucine plus isoleucine	3.5‡	15	<7.0		10.2	6.7–16.3
Serine plus glycine	10.0§	15	61.3	26.0–93.0	90.0	46.0–135.0
Taurine	5.0	15	24.1	13.0–31.0	52.0	28.4–100.0
Urea	1,000.0	15	11,450.0	3,800.0–24,000.0		

* Stein's values, 8 subjects (19).
† Includes the methyl histidines.
‡ Leucine, 2.0 mmoles; isoleucine, 1.5 mmoles.
§ Serine, 3.0 mmoles; glycine, 7.0 mmoles.

sample was between that of the 0.5 and 1 mM alanine standard, the dried residue from the urine was dissolved in 0.5 ml of the above described Tergitol-Safranin dye solution; where the intensity was between the 1 and 2 mM standard, it was dissolved in 1 ml of the Tergitol-Safranin solution; and where it was between the 2 and 4 mM standards, it was dissolved in 2 ml of the solution.

Results showed that the salts and other substances that dissolved in the Tergitol-Safranin solution increased its volume approximately 20 per cent. This increase in volume was not measured in each case but was assumed, and in the calculations appropriate corrections were made.

Chromatography. The developing solutions and staining reagents were the same as used in our earlier method for plasma amino acid determinations except for the following: *a*) 2, 4 and 8 μl aliquots of samples and 2, 3, 4, 6 and 8 μl aliquots of standard solution were placed on the chromatograms; *b*) S and S paper no. 589 (green ribbon) was used for the amino acid analysis determined by the ninhydrin-collidine and the isatin reagents; *c*) the determination of glycine with Solvent B (1) was omitted, since quantitative estimates made from chromatograms developed in this solvent have been found to be poor; furthermore, when taurine is present in large amounts, it interferes with the glycine zone. *d*) *p*-Dimethylaminocinnamaldehyde was used in lieu of *p*-dimethylaminobenzaldehyde in Ehrlich's reagent (15); and *e*) all periods of 16 hour development, except for the paper used to determine histidine and tyrosine, were reduced to 6 hours. It was found that substances on the chromatograms gave more intense stains after a 6 hour development period than after a 16 hour period; this modification, however, was a compromise since leucine and isoleucine were not resolved separately in the shorter time interval. The composition of the standard amino acid solution used for plasma, red cells and white cells is given in Table I; that for the urine amino acid determinations is given in Table II.[3] To minimize deterioration of the standard solution, 5 ml aliquots were freeze-dried and stored at −5° C. Glutamine, which was not stable in the presence of other amino acids in the dried preparations, and urea, which caused difficulty in drying of the amino acid solutions, were added to the Tergitol-Safranin solution which was used to reconstitute dried aliquots of the standard at approximately monthly intervals.

Estimations of the concentrations of the compounds were as previously described (1). The taurine concentration in the white cells was estimated by using the serine and glycine zone of the standard. The concentration of arginine, which has the same Rf value as taurine,

[3] All compounds were obtained from Mann Research Laboratories, New York, N. Y., except urea which was obtained from Merck and Co., Rahway, N. J., and ethanolamine which was obtained from Fisher Scientific Co., New York, N. Y. The compounds were dried *in vacuo* prior to weighing.

was found to be very low in the cells and its contribution to the intensity of the taurine zone was negligible; this was always confirmed by the Sakaguchi test. The concentration of O-phosphoethanolamine, which in Solvent A (1) has an Rf value approximately one-half that of glutamic acid, was estimated on the chromatograms developed 64 hours using the glutamic acid zone as a standard. Ergothioneine concentration was estimated on the papers treated with Pauly's reagent; its Rf value was slightly faster than histidine. Ethanolamine and β-amino isobutyric acid concentrations were estimated on the papers treated with the ninhydrin-collidine reagent after 6 hours' development. The Rf value of ethanolamine was slightly faster than valine; the Rf value of β-amino isobutyric acid was slightly faster than alanine. The concentration of guanidoacetic acid was determined by the Sakaguchi test; its Rf value was slightly faster than arginine. Glutathionine and diglutathionine, both major ninhydrin-positive components of erythrocytes, did not move from the origin of application on chromatograms developed in Solvent A and were not determined. Histidine and methyl histidines (the latter are present in moderate amounts in urine) are reported as a combined value, since the isopropanol solvent does not resolve these compounds.

To express the amino acid values on the basis of a kilogram of water, plasma was taken to be 92 per cent and leukocytes 74 per cent water by volume. The erythrocytes were taken to be 65 per cent and urine 100 per cent water by weight.

Statistics. The individual amino acid values found in the study of the plasma, erythrocytes and leukocytes were assumed to be normally distributed. This assumption was reasonably satisfactory in most instances. In some leukocyte analyses, a skew distribution was evident for substances in low concentration. No attempt was made to adjust for this, since its effect was never such as to invalidate any of the conclusions drawn. Skewness was of such magnitude in some of the urine studies that ranges of the excretion rates were given rather than an expression of variability by a standard deviation notation.

The test statistic $t = \frac{\bar{x} - \bar{y}}{\sqrt{s_x^2/n_x + s_y^2/n_y}}$ was used to determine whether the mean of one sample population was equal to the mean of another sample population (16). The degree of freedom for this test statistic (referred to hereafter as the t test) was obtained from the formula:

$$\frac{(s_x^2/n_x + s_y^2/n_y)^2}{\frac{s_x^4/n_x^2}{(n_x + 1)} + \frac{s_y^4/n_y^2}{(n_y + 1)}} - 2$$

where s_x and n_x were, respectively, the standard deviation and number of samples from population x; and s_y and n_y were the same parameters from population y. The level of significance, α, that is, the probability of rejecting the hypothesis that the two means were equal when in fact they were equal, was taken as 0.05 for a two-sided test. Since in some categories the number of subjects was small, the β error, which is the probability that the significant difference would not be redetected, in another set of experiments in which the same number of samples was employed, was large. The β error has therefore been estimated for analyses where significant differences have been found.

The sample correlation coefficient for regression analysis, R, was calculated to detect associations between concentrations intracellularly and extracellularly. Association probabilities greater than 0.95 (a rejection of $p = O$ at an α level of 0.05) are noted in the tables by the symbol "+." The symbol O indicates a failure of this test to detect an association.

RESULTS AND DISCUSSION

Reliability of the analytical methods

The precision of analysis and recovery of added amino acids have been previously studied for plasma (1). Investigations as to the suitability of the salt-saturated chromatographic technique for the analysis of erythrocytes, leukocytes and urine are reported here.

Recovery of amino acids added to lysed erythrocyte solutions and variability of the erythrocyte analyses. Amino acids added to the lysed erythrocyte solutions were recovered quantitatively in a 2 hour period of dialysis (Table III). When the duration of dialysis was extended up to 16 hours, variable increases in the concentration of amino acids in the dialysate were observed. For 2 hours of dialysis this amounted to less than 10 per cent and was not corrected for. The increase appears to be partly enzymatically induced, since it could be diminished by addition of enzyme inhibitors such as dodecyl sulfate, EDTA and N-maleylimide to the lysed erythrocyte solution prior to dialysis. The accuracy of the erythrocyte analyses as computed from the average variability of the data in Table III, was similar to that of the plasma analyses (1).

The red cell samples were found to be slightly contaminated with white cells. By comparison of the taurine levels found in the red cells with those found in the white cells, and assuming that taurine was absent in red cells, it was shown that this contamination could not increase the red cell levels of the other substances determined by more than 2 per cent.

Reproducibility of leukocyte analyses. In view of the small amounts of solution available, paper chromatography proved to be a very useful tech-

TABLE III

Recovery of amino acids added to lysed erythrocyte solutions

	Average amts. added*	Total amts. calc. present*	Total amts. found*	Recovery
	μmoles/kg water			%
Alanine	601	972	1,010	106
α-Amino-*n*-butyric acid	72	102	110	111
Glutamic acid	313	1,066	1,180	136
Glutamine	970	1,700	1,830	113
Histidine	243	360	387	111
Lysine	315	487	415	77
Methionine	72	102	92	86
Ornithine	193	336	350	107
Phenylalanine	120	191	162	76
Proline	340	468	423	87
Threonine	315	446	420	92
Tryptophan	77	95	82	83
Tyrosine	169	241	220	88
Valine	435	667	645	95
Leucine plus isoleucine†	362	565	557	98
Serine plus glycine‡	726	1,553	1,680	117
Urea	9,700	16,360	16,700	103

* The mean of duplicate analyses.
† Leucine 217; isoleucine 145.
‡ Serine 242; glycine 484.

TABLE IV

Reproducibility of unbound amino acid analyses of lysed leukocytes **

	Average of means	Standard deviation	Coefficient of variation	Coefficient of variation of plasma analyses†
	μmoles/kg water			
Alanine	2,180	143	7	5.2
Glutamic acid	3,720	560	15	9.4
Glutamine	635	131	8	6.2
Histidine	374	83	22	5.9
Lysine	1,300	78	6	8.1
Methionine‡	540	47	9	18.0
Ornithine	1,300	163	13	11.0
Phenylalanine‡	730	78	11	12.0
Proline	648	134	21	8.7
Threonine	1,190	185	16	10.8
Tryptophan‡	160	23	14	18.0
Tyrosine	392	73	18	10.0
Valine	873	115	13	4.0
Ethanolamine‡	148	57	38	
Leucine plus isoleucine	1,320	111	9	7.5
Serine plus glycine	6,084	840	14	7.0
Taurine	27,800	937	4	
O-phospho-ethanolamine	7,940	446	6	

* Lysed cells of two subjects were analyzed, each in triplicate. Four periods of dialysis were for 2 hours, two periods were for 4 hours.
† See reference (1).
‡ Three determinations only.

nique for the analysis of unbound amino acid concentrations in the leukocytes. The amino acid values obtained from the lysed leukocyte solutions, as opposed to the values obtained from the lysed erythrocyte solutions, were found not to change significantly on extension of the dialysis period from 2 to 4 hours; furthermore, duplicate analyses of the leukocytes were only slightly less reproducible than those of plasma analyses (Table IV). This stability of the leukocyte suspensions was surprising, since these cells are generally considered to have more active proteolytic enzymes than the erythrocytes. Undoubtedly an important factor in this instance was the relative volume of lysed cells dialyzed; the volumes of the leukocytes were 1/20 to 1/40 the volume of the erythrocytes.

Effect of temperature on the concentrations in the cells and plasma. Since it was desirable for stability purposes to chill the blood to 2° C for its transportation to the laboratory and separation of the cells, it became necessary to obtain some estimate of the effect of cooling on the amino acid distributions. Whole blood was drawn and the plasma and cells separated as rapidly as possible at 37° C. This posed no difficulty for the plasma and erythrocytes, since separations were made within 30 minutes after drawing the blood by increasing the centrifugal force to 2,500 × G. For the leukocytes, centrifugation of the dextran supernatant solution at 37° C gave a packed cell plug which was frequently irreversibly clumped even though the time of centrifugation was reduced; in 12 attempts only 3 samples redispersed. The results of these studies are shown in Table V.

With the exception of two compounds, ornithine in the plasma and phenylalanine in the erythrocytes, there were no significant differences as evaluated by the t test in the analyses conducted at 2° and 37° C. The values for ornithine in the plasma and phenylalanine in the erythrocytes differed by amounts which were at the border line of significance ($\alpha = 0.05$). Furthermore the β error was large because of the small

TABLE V

Concentrations of amino acids in plasma, erythrocytes and leukocytes at 37° C *

	A Plasma		B Erythrocytes		C Erythrocyte-plasma ratio		D Leukocytes	
	Mean	PM†	Mean	PM	Mean	PM	Mean	PM
Alanine	398 ± 27‡	ns	477 ± 30	ns	1.22 ± 0.30	ns	2,860 ± 940	ns
α-Amino-*n*-butyric acid	25 ± 5	ns	26 ± 1	ns	1.14 ± 0.40	ns		
Arginine	57 ± 3	ns						
Glutamic acid			465 ± 33	ns			2,520 ± 670	ns
Glutamine	421 ± 60	ns	477 ± 34	ns	1.17 ± 0.26	ns	1,660 ± 100	ns
Histidine	87 ± 12§	ns	121 ± 21	ns	1.35 ± 0.52§	ns	950‖	ns
Lysine	210 ± 18	ns	202 ± 10	ns	1.00 ± 0.26	ns	1,910 ± 540	ns
Methionine			51 ± 9	ns				
Ornithine	135 ± 20	SH(0.50)	202 ± 20	ns	1.50 ± 0.40	ns	2,170 ± 520	ns
Phenylalanine	67 ± 7	ns	82 ± 9	SH(0.50)	1.26 ± 0.21	ns		
Proline	200 ± 14	ns	226 ± 17	ns	1.15 ± 0.12	ns		
Threonine	179 ± 11	ns	183 ± 8	ns	1.04 ± 0.18	ns	1,740 ± 370	ns
Tryptophan			35 ± 4	ns				
Tyrosine	80 ± 3	ns	82 ± 6	ns	1.03 ± 0.10	ns		
Valine	205 ± 11	ns	221 ± 16	ns	1.08 ± 0.16	ns	1,010 ± 420	ns
Ergothioneine	<10		508 ± 160	ns			<300	
Ethanolamine	<10		<10				<300	
Leucine plus isoleucine	233 ± 8	ns	296 ± 19	ns	1.29 ± 0.17	ns	2,860‖	ns
Serine plus glycine	687 ± 66	ns	1,091 ± 77	ns	1.62 ± 0.28	ns	11,900 ± 2,450	ns
Taurine			<70				36,400 ± 8,240	ns
Urea	5,413 ± 190	ns	5,887 ± 600	ns	1.09 ± 0.18	ns		
O-phospho-ethanolamine							3,550 ± 1,540	ns
Cell volume¶							0.501 ± 0.121	ns

* The plasma and erythrocyte values are the mean from four subjects. The leukocyte values are the mean of analyses of three subjects. The low yields of leukocytes at this higher temperature did not give sufficient material to determine several of the amino acids. The values are expressed as μmoles per kilogram water.

† Comparison of the mean at 37° C with the appropriate mean at 2° C taken from Table I, VI, or VII; ns = not significantly different; S = significantly different; H or L = a high or low value, respectively, at 37° C. The number in the parentheses is the β error.

‡ Standard deviation of the mean.

§ Three determinations.

‖ One determination.

¶ Volume of one billion leukocytes in milliliters.

number of samples determined. To investigate further whether these concentration differences were real, additional ornithine and phenylalanine determinations were made at 2° and 37° C with blood from the same donor. In this instance the values at the two temperatures agreed in duplicate analyses within 15 per cent, which is well within the experimental variability of the method and makes it unlikely that the differences found before were significant. Given as micromoles per kilogram water, ornithine was 118, at 2° and 113 at 37° C, phenylalanine was 50 at 2° and 58 at 37° C. To obtain more power in our statistical treatment, a sign-rank test was conducted on all the data. The analyses were normalized by dividing the difference in the means at the two temperatures by the standard deviation. By this test the results of the plasma and leukocyte analy-

ses were found to be identical at the two temperatures whereas the erythrocyte determinations were significantly higher at 37° than at 2° C. Further evidence of higher values for the erythrocytes at 37° C was seen by comparison of the totals of the amino acid concentrations in which the values at 37° were 7 per cent higher than those at 2° C. In that the plasma amino acid values did not differ whether determinations were conducted at the higher or lower temperature, it can be inferred that the changes in the erythrocyte concentrations were not induced by a distributional shift but, rather, that they were due to the lower stability of the erythrocytes at 37° C.

From these results it was concluded that chilling the blood to 2° C for up to 2 hours would not cause major changes in the unbound amino acid distributions.

Reliability of the urine amino acid determinations. The excretion rates of most compounds in urine vary from large amounts to nil. This, combined with the unusually large number of compounds which may be excreted (17–19) leads to some uncertainty as to whether the amino acid zones on the chromatograms were pure zones or zones in which the amino acid was mixed with other substances. Several steps in the analytical procedure tended to reduce this uncertainty: *a*) the passage of chilled urine through a short resin column (described above) removed all colored and cold-insoluble solid material, and presumably other substances, but not the amino acids or urea; *b*) the characteristic colors shown by many of the amino acids with the ninhydrin-collidine reagent (1) helped greatly in preventing false identification of substances; and *c*) the variety of staining reagents used in each analysis assisted in identifying zones that contain mixtures of substances. Furthermore, in some instances, zones were rechromatographed in different solvents to determine whether they contained mixtures of compounds. An important factor with the salt-saturated systems was the tendency for a large number of unknown substances to move with slow Rf values and not to be encountered in the regions of the amino acid zones. Exceptions to this were the slow moving amino acids, aspartic and glutamic. In about one-third of the urines, substances interfered with the analysis of these two amino acids. In the remaining urines no color zones were found at glutamic acid and aspartic acid locations. Whereas paper chromatography has certain drawbacks for urine analyses, it does not lead to the destruction of glutamine, tryptophan or possibly other labile substances which are destroyed by conditions used in resin column analyses (19).

A recovery study of the substances present in the urine standard added to urine specimens indicated no loss during the procedure. Recoveries of 83 to 132 per cent were obtained. The coefficient of variation averaged about 7, which is similar to the accuracy obtained in the other analyses using salt-saturated paper chromatography.

Normal fasting values in the body fluids

Unbound amino acid concentrations in plasma. The concentrations of unbound amino acids found in plasma are reported in Table I. These values agree reasonably well with those previously reported in which larger aliquots were used for analysis, except that the combined values of serine and glycine in the plasma are higher than those reported previously (1). Plasma values for tryptophan are not given. Tryptophan was largely bound by the plasma albumin and the conditions under which the present experiments were conducted would not give a correct unbound value for tryptophan.[4] One anomalously high glutamic acid value was found (70 μmoles per kg water); all other subjects had values lower than 30 μmoles per kg water.

Unbound amino acid concentrations in erythrocytes. The concentrations of the amino acids in the erythrocytes and the erythrocyte-plasma concentration ratios are given in Table VI. The means of the plasma and the erythrocyte concentrations were statistically the same for alanine, α-amino-*n*-butyric acid, glutamine, lysine, methionine, phenylalanine, proline, threonine, tyrosine, valine, leucine plus isoleucine, and urea. In testing the distribution of molecules which carry net charges, a Donnan ratio of 1.4 was used between the plasma and erythrocytes. The average of the erythrocyte-plasma ratios for these substances, excluding that of methionine whose ratio was high and irregular, was 1.10. The increase over the expected ratio of 1.0 is believed to be an artifact induced by enzymatic activity in the erythrocytes

[4] McMenamy, R. H., and Lund, C. C. To be published.

TABLE VI

Unbound amino acid concentrations in erythrocytes

	Erythrocytes		Erythrocyte-plasma ratio			
	No. of subjects		No. of subjects	Mean	p_M*	p_C†
		μmoles/kg water				
Alanine	15	427 ± 25‡	15	1.08 ± 0.04	ns	+
α-Amino-*n*-butyric acid	9	25 ± 2	9	0.96 ± 0.08	ns	+
Arginine	11	<12	10	low	SL(<0.01)	ID§
Glutamic acid	15	374 ± 33	15	high	SH(<0.01)	ID§
Glutamine	15	601 ± 46	15	1.27 ± 0.10	ns	0
Histidine	14	131 ± 8	12	1.58 ± 0.12	SH(0.10)	0
Lysine	15	233 ± 24	15	1.15 ± 0.09	ns	0
Methionine	12	30 ± 4	12	1.63 ± 0.34	ns	0
Ornithine	14	185 ± 13	11	2.50 ± 0.11	SH(<0.01)	0
Phenylalanine	14	59 ± 4	14	1.18 ± 0.28	ns	0
Proline	15	191 ± 11	15	1.13 ± 0.06	ns	+
Threonine	15	157 ± 9	15	0.97 ± 0.06	ns	0
Tryptophan	15	21 ± 4		(1.2)‖		
Tyrosine	15	72 ± 5	15	1.03 ± 0.08	ns	+
Valine	15	223 ± 12	15	1.12 ± 0.05	ns	+
Ergothioneine	13	458 ± 51	14	high	SH(<0.01)	ID§
Ethanolamine	15	<10				
Leucine plus isoleucine	14	211 ± 21	13	1.16 ± 0.09	ns	+
Serine plus glycine	14	948 ± 48	14	1.59 ± 0.10	SH(0.01)	0
Taurine	8	<70				
Urea	13	6,000 ± 370	13	1.14 ± 0.05	ns	+

* A comparison of the mean value in the erythrocytes to the mean value in the plasma; see footnote † Table V for terms.

† Probability that there is a correlation between the concentration in the erythrocytes and the concentration in the plasma; + = correlation indicated, 0 = correlation not supported.

‡ Standard deviation of the mean.

§ Indeterminate, concentration of substance in one medium is missing.

‖ Estimated; see text.

(see previous discussion); and, if such an increase is allowed, the probability of equivalence of means of the above list of compounds is further improved. On the other hand, the mean concentrations of arginine, glutamic acid, histidine, ornithine, ergothioneine, and serine plus glycine were significantly different, implying that a concentration gradient across the red cell membrane exists for many substances. Aspartic acid was also undoubtedly concentrated by the erythrocytes; it was frequently seen on the chromatograms of the red cell dialysates but never on those of the plasma. With the possible exception of histidine, concentration gradients of similar magnitude have been observed by others (8, 9, 20, 21). Tryptophan, in contrast to the plasma studies, was not bound to a nondialyzable component of lysed red cells (see Table III) and, therefore, concentrations obtained for it by dialysis were reliable. The erythrocyte-plasma ratio for the unbound tryptophan concentration was estimated by using an average unbound plasma tryptophan value of 17 μmoles per kg water.[4]

The low concentrations of arginine and the elevated levels of ornithine in red cells are of interest in view of the presence of arginase in these cells (22). The observed concentration gradients may be due to the slow transport of these compounds

across the red cell membrane in the presence of relatively high arginase activity. Such a phenomenon as this had been attributed to Ehrlich ascites carcinoma cells by Johnstone (23).

The hypothesis: "Is the concentration of the substance in the erythrocytes influenced by the concentration in the plasma?" was examined by computation of the correlation coefficient R (see column p_C, Table VI). A correlation with a probability $\geqslant 0.95$ was found for alanine, α-amino-*n*-butyric acid, proline, tyrosine, valine, leucine plus isoleucine, and urea implying that a concentration dependence exists between the two media. An association in concentration intracellularly and extracellularly was not found for several of the compounds; however, by the very nature of the mathematical test this cannot be taken as an index of no dependence. Furthermore, in this investigation normal fasting subjects were studied and no large fluctuation in concentrations was imposed which would have made the correlation tests more sensitive. A detailed study of the relationship between the erythrocyte-plasma concentrations is in progress.

Unbound amino acid concentration in leukocytes. The amino acid concentrations in the leukocytes of normal subjects were considerably higher than those in plasma or erythrocytes (Table VII). When compared with plasma glutamic acid, ornithine, serine plus glycine, taurine, and O-phosphoethanolamine were concentrated 20 times or more by the leukocytes, whereas the other amino acids (excluding arginine) were concentrated on the average 9.9 times (range 4.8 to 14.5). It was of interest that glutamic acid, ornithine, and serine plus glycine also showed an enhanced concentration relative to the other amino acids in the erythrocytes, and it is implied that the concentrative potential for both the erythrocytes and leukocytes is large for these substances. Arginine was generally low in the leukocytes, although an occasionally high result was found, and ornithine, as noted above, was elevated. This again may be due to the arginase which has been found in the leukocytes (24). α-Amino-*n*-butyric acid was probably present in the leukocytes; its quantity was not estimated due to the interference on the chromatograms of an unknown ninhydrin-positive substance. The leukocyte-plasma ratio for tryptophan was estimated by using an average plasma concentration for tryptophan of 17 μmoles per kg water.[4] It also should be noted that whereas there was a direct correlation between the concentration of many amino acids in plasma and in erythrocytes, this was not observed in the leukocyte-plasma distribution (see column p_C, Table VII). This may well be due to the small difference in concentration found in bloods of subjects in the fasting state.

The concentration of amino acids in the leukocytes is of the approximate order of magnitude found in other mammalian tissues (25, 26). This is not unexpected, since the leukocytes appear to perform most of the general metabolic functions of such cells.

Unbound amino acid excretion rates in urine. Williams and co-workers (18) found that the 24-hour excretion patterns of amino acids were reasonably constant from day to day in any one subject. We studied the excretion rate of two fasting individuals by means of urines collected for shorter periods on different days and also found for each of these that the rates were reasonably constant from day to day (Table VIII). With the exception of taurine, the mean variation was less than 40 per cent. This variation is small in view of the large differences in excretion rates of urines from different individuals (see later results). The excretion rates were not related to the volume of urine voided during the period.

The effect of food ingestion on excretion is shown by comparison of columns A and C of Table IX. When subjected to a sign-rank test the differences between these columns were highly significant. All substances were consistently excreted at a higher rate in the nonfasting period, averaging 60 per cent higher than in the fasting period. This was in qualitative agreement with α-amino nitrogen values of 6.6 mg per hour for nonfasting individuals, and 4.4 mg per hour for fasting individuals reported by Eckhardt, Cooper, Faloon and Davidson (27). The increased excretion in the nonfasting period was approximately the same for each amino acid. In unpublished experiments it has been found that the unbound amino acids increased an average of 20 per cent in the plasma of five normal adults 2 hours after a typical 25 g protein meal. This suggests that

TABLE VII

Unbound amino acid concentrations in leukocytes

	Leukocytes		Leukocyte-plasma ratio			
	No. of subjects		No of subjects	Mean	p_M*	p_C†
		μmoles/kg water				
Alanine	15	2,881 + 256‡	15	7.5 ± 0.9‡	SH	0
Arginine	13	<290 ± [46]	13	<4.2 ± [1.2]	ID§	
Glutamic acid	15	2,745 ± 251		very high	SH	
Glutamine	15	2,650 ± 251	15	5.2 ± 0.6	SH	0
Histidine	10	762 ± 70	9	9.8 ± 1.5	SH	0
Lysine	15	2,111 ± 216	15	10.2 ± 1.1	SH	0
Methionine	12	391 ± 54	11	14.5 ± 2.1	SH	0
Ornithine	12	1,767 ± 113	9	20.6 ± 2.0	SH	0
Phenylalanine	9	647 ± 105	8	13.6 ± 2.3	SH	0
Proline	15	862 ± 79	15	4.8 ± 0.4	SH	0
Threonine	14	2,345 ± 174	13	14.4 ± 1.3	SH	0
Tryptophan	10	222 ± 31		(11.7)‖	SH	
Tyrosine	15	480 ± 97	15	12.0 ± 1.2	SH	0
Valine	15	1,335 ± 132	15	6.6 ± 0.6	SH	0
Ergothioneine	15	<300				
Ethanolamine	15	<250				
Leucine plus isoleucine	13	1,999 ± 195	12	10.4 ± 1.2	SH	0
Serine plus glycine	15	13,021 ± 1,480	14	22.1 ± 2.8	SH	0
Taurine	15	28,683 ± 2,726		very high	SH	
O-phospho-ethanolamine	8	2,651 ± 389		very high	SH	
Cell volume¶	13	0.604 ± 0.028				

* A comparison of the mean in the leukocytes and plasma; see footnote † Table V for terms. The β error is less than 0.01 in all instances.

† Probability that there is a correlation between the concentration in the leukocytes and the concentration in the plasma; + = correlation indicated, 0 = correlation not indicated.

‡ Standard deviation of the mean. Values in brackets are approximate.

§ Indeterminate, concentration of substance in one medium was missing.

‖ Estimated; see text.

¶ Volume of one billion leukocytes.

the increased excretion is caused by the postprandial rise in the plasma amino acids. The fasting urine specimens collected as described in the experimental procedure should be largely free of this diet effect. Excretion rates thus measured should be more directly related to the fasting blood amino acid levels than are specimens collected through a 24 hour period which encompasses fasting and nonfasting intervals.

In column B of Table IX the excretion rates of subjects undergoing moderate exercise (an hour's walk of 3 to 4 miles between the hours of 8 and 10 a.m.) are given. This was an attempt to see whether such activity influenced the amino acid excretion. The differences between the overnight specimens and these latter samples were not significant. The similarity of the excretion rates of the two periods also implied that a 5 hour interval after the meal was sufficient to obtain constant fasting values. Diurnal variations were not studied in this work.

The urine excretion rates of the 15 normal fasting subjects on whom the blood analyses were conducted are presented in Table II. For comparison, Stein's values obtained by resin column chromatography on 24-hour collections of nonfasting

TABLE VIII

Urinary excretion of amino acids of two normal subjects on different days

	Subject A			Subject B		
	Day 1	Day 10	Day 135	Day 1	Day 10	Day 135
		μmoles/hr			*μmoles/hr*	
Alanine	11.0	12.0	9.8	11.3	8.4	7.7
Glutamine	28.0	38.0	26.3	27.8	22.0	26.9
Histidine plus methyl histidines	64.0	81.6	62.5	56.4	42.0	42.3
Lysine	9.0	18.0	9.9	7.0	4.2	8.7
Threonine		13.2	8.2		5.2	
Tryptophan	<2.0	4.2	<2.0	<2.0	1.1	
Tyrosine	3.5	3.6	<2.0	3.5	5.2	
Serine plus glycine	79.0	114.0	66.0	155.0	105.0	96.0
Taurine	17.0	26.0	13.1	11.3	14.7	4.2
Volume of urine excreted, ml/hr	139	120	164	70.5	52.5	23

subjects are included in the table (19). The generally higher values reported by Stein were probably due to the analyses of nonfasting specimens. In both studies there was considerable variation in the excretion rates of different subjects. This variability was noted previously by Williams and colleagues (18) and was used as a basis for demonstrating individual differences. The amino acids frequently found excreted in high amounts in normal urines were taurine, threonine, glycine plus serine, histidine plus methyl histidines, alanine, glutamine and lysine. Excretion rates of certain other amino acids (proline, leucine, isoleucine, arginine, glutamic acid, aspartic acid, valine, α-amino-*n*-butyric acid, and phenyalanine) were usually low in the fasting specimens of normal individuals. These results agree qualitatively with those of other workers (17–19, 28).

TABLE IX

The effect of food intake and exercise on the urinary excretion of amino acids *

	Fasting A Midnight–7 a.m.	Exercise (fasting) B 8–10 a.m.	Nonfasting C 10 a.m.–midnight
Alanine	10.7 ± 0.8†	11.4 ± 0.9	22.0 ± 2.7
Glutamine	29.1 ± 3.5	34.2 ± 1.1	47.8 ± 7.8
Histidine plus methyl histidines	61.0 ± 8.3	59.9 ± 3.6	85.0 ± 13.0
Lysine	9.8 ± 3.0	8.0 ± 1.3	13.0 ± 2.9
Threonine	<8.5	<9.8	13.7 ± 3.5
Tryptophan	<1.1	<1.9	3.4 ± 1.2
Tyrosine	4.2 ± 0.4	6.0 ± 0.9	8.7 ± 1.0
Serine plus glycine	114.0 ± 16.0	138.0 ± 20.0	180.0 ± 12.0
Taurine	17.4 ± 3.3	26.0 ± 2.6	30.6 ± 7.2
Volume of urine excreted, ml/hr	96.0	106.0	86.0

* Averages of two experiments on each of two subjects. The meals eaten were their usual ones and contained on each day at least 75 g of protein. The values are expressed as micromoles excreted per hour.

† The standard deviation of the mean. Alanine, tyrosine, and serine plus glycine were on the basis of the *t* test significantly higher in column C than A. When compared by the sign-rank test columns A and B were not significantly different, whereas columns A and C were different at a high level of significance ($\alpha = 0.008$).

SUMMARY

1. The concentrations of unbound amino acids in the plasma, erythrocytes and leukocytes, and rates of excretion of amino acids in the urine of normal human subjects have been determined using salt-saturated paper chromatography for analysis. The variability of the determinations and the effect of temperature on the distribution of the cell components have also been investigated.

2. The mean concentrations of values in the plasma and erythrocytes for alanine, α-amino-*n*-butyric acid, glutamine, methionine, phenylalanine, proline, threonine, tyrosine, valine, leucine plus isoleucine, and urea did not differ significantly. On the other hand the mean concentration of arginine, glutamic acid, histidine, ornithine, serine plus glycine, and undoubtedly aspartic acid were significantly different in the plasma and the erythrocytes. When examined by a linear regression analysis a positive correlation for an association in concentration between the erythrocytes and plasma was found for alanine, α-amino-*n*-butyric acid, proline, tyrosine, valine, leucine plus isoleucine, and urea. This supports a diffusion-controlled distribution for these latter substances.

3. The concentrations of amino acids in the leukocytes, with the exception of arginine which was generally too low to be measured, were much higher than those found in the plasma. The leukocyte-plasma ratios ranged from 4.8 to 14.5 for most of the amino acids. Glutamic acid, ornithine, and combined serine plus glycine were concentrated in leukocytes at leukocyte-plasma ratios of 20 or higher. Taurine and O-phosphoethanolamine, which were in very high concentration in the leukocytes, were not found in the plasma analysis, and only small concentrations of taurine were observed in the red cells. A linear regression analysis showed no relationship between the concentrations of any of the amino acids in the plasma and leukocytes.

4. In the urine studies a reasonably constant excretion rate for the same individual was observed from day to day, whereas a large variation was found in the excretion rates among different individuals. The excretion rates of amino acids during a nonfasting period were on the average 60 per cent higher than during the fasting period. Exercise of a moderate extent had little effect on the excretion rates.

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REFERENCES

1. McMenamy, R. H., Lund, C. C., and Oncley, J. L. Unbound amino acid concentrations in human blood plasmas. J. clin. Invest. 1957, **36**, 1672.
2. Stein, W. H., and Moore, S. The free amino acids of human blood plasma. J. biol. Chem. 1954, **211**, 915.
3. Levenson, S. M., Howard, J. M., and Rosen, H. Studies of the plasma amino acids and amino conjugates in patients with severe battle wounds. Surg. Gynec. Obstet. 1955, **101**, 35.
4. Stein, W. H., Bearn, A. G., and Moore, S. The amino acid content of the blood and urine in Wilson's disease. J. clin. Invest. 1954, **33**, 410.
5. Iyer, G. Y. N. Paper-chromatographic study of free amino acids in normal and anaemic erythrocytes. Indian J. med. Res. 1957, **45**, 213.
6. Nour-Eldin, F., and Wilkinson, J. F. Amino-acid content of white blood cells in human leukemias. Brit. J. Haemat. 1955, **1**, 358.
7. Rouser, G. Free or easily extractable amino acids in blood cells and body fluids *in* The Leukemias: Etiology, Pathophysiology and Treatment. J. W. Rebuck, F. H. Bethell and R. W. Monto, Eds. New York, Academic Press, 1957, p. 361.
8. Christensen, H. N., Cooper, P. F., Jr., Johnson, R. D., and Lynch, E. L. Glycine and alanine concentrations of body fluids; experimental modification. J. biol. Chem. 1947, **168**, 191.
9. Johnson, C. A., and Bergeim, O. The distribution of free amino acids between erythrocytes and plasma in man. J. biol. Chem. 1951, **188**, 833.
10. Lund, C. C., McMenamy, R. H., and Neville, G. J. A syringe attachment for decalcification of small quantities of blood. Amer. J. clin. Path. 1957, **28**, 328.
11. Tullis, J. L., and Rochow, E. G. Non-wettable surfaces. Blood 1952, **7**, 850.
12. Skoog, W. A., and Beck, W. S. Studies on the fibrinogen, dextran and phytohemagglutinin methods of isolating leukocytes. Blood 1956, **11**, 436.
13. McMenamy, R. H., and Oncley, J. L. The specific binding of L-tryptophan to serum albumin. J. biol. Chem. 1958, **233**, 1436.
14. Gold, G. L., and Solomon, A. K. The transport of sodium into human erythrocytes *in vivo*. J. gen. Physiol. 1955, **38**, 389.
15. Mason, J. H., and Archer, A. A. P. G. Use of

p-dimethylaminocinnamaldehyde as spray reagent for indole derivatives on paper. Biochem. J. 1958, **69**, 60P.

16. Bowker, A. H., and Lieberman, G. J. Handbook of Industrial Statistics. Englewood Cliffs, N. J., Prentice-Hall, 1955, p. 872.
17. Westall, R. G. The amino acids and other ampholytes of urine; unidentified substances excreted in normal human urine. Biochem. J. 1955, **60**, 247.
18. Williams, R. J., Berry, H. K., and Cain, L. Biochemical Institute Studies IV. Univ. of Texas Publication, no. 5109, May 1, 1951, pp. 7, 70, 150.
19. Stein, W. H. A chromatographic investigation of the amino acid constitutents of normal urine. J. biol. Chem. 1953, **201**, 45.
20. Melville, D. B., and Lubschez, R. A method for the determination of ergothioneine in blood. J. biol. Chem. 1953, **200**, 275.
21. Jocelyn, P. C. The distribution of ergothioneine in blood as determined by a new method of estimation. Biochem. J. 1958, **70**, 656.
22. Covolo, G. C., and West, R. The activity of arginase in red blood cells. J. clin. Endocr. 1947, **7**, 325.
23. Johnstone, R. M. Arginine uptake and arginase activity in Ehrlich ascites carcinoma cells. Canad. J. Biochem. 1959, **37**, 589.
24. Borghetti, A., and Scarpioni, L. Arginase activity of human leukocytes. Enzymologia 1956, **17**, 338.
25. Tallan, H. H., Moore, S., and Stein, W. H. Studies on the free amino acids and related compounds in the tissues of the cat. J. biol. Chem. 1954, **211**, 927.
26. Piez, K. A., and Eagle, H. The free amino acid pool of cultured human cells. J. biol. Chem. 1958, **231**, 533.
27. Eckhardt, R. D., Cooper, A. M., Faloon, W. W., and Davidson, C. S. The urinary excretion of amino acids in man. Trans. N. Y. Acad. Sci. 1948, **10**, 284.
28. Doolan, P. D., Harper, H. A., Hutchin, M. E., and Shreeve, W. W. Renal clearance of eighteen individual amino acids in human subjects. J. clin. Invest. 1955, **34**, 1247.

UNBOUND AMINO ACID CONCENTRATIONS IN PLASMA, ERYTHROCYTES, LEUKOCYTES AND URINE OF PATIENTS WITH LEUKEMIA *

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The distribution of unbound amino acids in the plasma and blood cells of patients with leukemia may be expected to reflect abnormalities in both the concentrating ability of the cells for the amino acids and in the amino acid metabolism. The distribution of unbound amino acids in the plasma and blood cells of normal subjects has been reported in an earlier communication (1). Studies of the distribution of unbound amino acids in leukemic bloods do not appear to have been previously made, although Kelley and Waisman (2) have studied plasma concentrations and Nour-Eldin and Wilkinson (3) have studied the relative concentrations of several amino acids in the leukocytes of leukemic patients. Some qualitative variations in amino acid concentrations in leukocytes from patients with different types of leukemia have also been reported by Rouser (4).

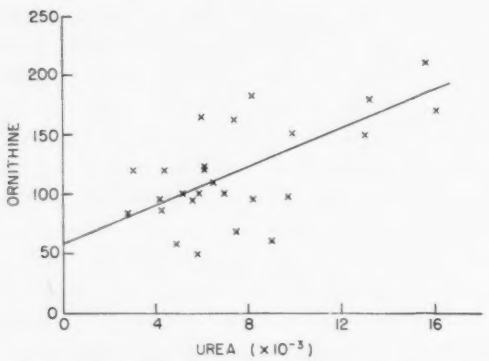

FIG. 1. COMPARISON OF CONCENTRATIONS OF ORNITHINE AND UREA IN PLASMA OF LEUKEMIC PATIENTS. Micromoles per kilogram of water; line drawn by least squares.

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METHODS

The methods used for collection of the blood and the urine specimens, and for the analysis of the unbound amino acids were the same as those used for normal subjects (1). All bloods were drawn at the end of an approximate 14 hour fast, and all urines were collected during the last 8 hours of this period.

The notations used for expressing the variability of the data and for expressing the results from tests of different hypotheses are the same as those used previously. The level of significance, α, was established at 0.05. The β error was estimated for each evaluation where a significant difference was detected (1).

RESULTS

Unbound amino acid concentrations in plasma of leukemic patients

The analyses of 32 plasma specimens from 23 patients with various types of leukemia are given in Table I (see Case Histories; analyses L18 and M2 were lost). With the exception of arginine, to be discussed later, there were no differences in the plasma levels that could be associated with any particular type of leukemia or its treatment. To identify the patients with high or low plasma concentrations, the fourth, and fifth columns of Table I list, respectively, the analyses that were less than and greater than 2 SD from the mean found for normal subjects. This latter distinguishes the analyses which differ from the normal mean at approximately the 0.05 level of significance.

Both the ornithine and urea concentrations were found to be high in the leukemic plasmas. Except for one patient, J.W., the increase in ornithine concentration correlated very well with the increase in plasma urea values (see Figure 1). The product moment correlation coefficient, R, calculated for a correlation between ornithine and urea concentrations in plasma was found to be

TABLE I

Concentration of unbound amino acids in the plasma of leukemic patients

	No. of analyses	Mean	PM*	Analyses with values 2SD less than normal mean†	Analyses with values 2SD higher than normal mean†
		μmoles/kg water			
Alanine	32	336 ± 21‡	SL (0.40)	L13	G10
α-Amino-*n*-butyric acid	25	27 ± 3	ns	0	G13
Arginine§	27	68 ± 4	ns	0	0
Glutamic acid	32	<33	ns	0	G10, 12, 13
Glutamine	32	514 ± 15	ns	0	0
Histidine	30	89 ± 5	ns	0	0
Lysine	32	217 ± 9	ns	L13	0
Methionine	25	22 ± 2	ns	0	L11
Ornithine	29	121 ± 8	SH (0.01)	0	G10, 12, L3, 7, 8, 9, 15, 16, 17
Phenylalanine	31	65 ± 5	ns	0	G10, L7, 8, 9
Proline	32	201 ± 13	ns	0	G7, 13, 14
Threonine	30	167 ± 7	ns	L10a, 13	G2, 10, L5, 11
Tyrosine	32	71 ± 4	ns	M1, L11	G7, 10, 11, L4
Valine	32	199 ± 9	ns	M1	L4
Ethanolamine	32	<16	high	0	G4, 7, 8, 13, L3, 8, 13, 15
Leucine plus isoleucine	30	209 ± 14	ns	0	G7, 10, 13
Serine plus glycine	31	664 ± 42	ns	G3, 7, 10a	G2, 10, L7, 15
Urea	30	8,000 ± 877	SH (0.05)	G2, L10a	G10, 13, M1, L5–9, 13, 16, 17
β-Amino isobutyric acid	2	202‖	high	0	G13, L1

* Comparison of the mean value of the patients with the mean value of normal subjects (1); ns = not significantly different; S = significantly different; H and L indicate high and low values respectively, in the patient analyses; the β error is placed in parentheses.

† Taken from reference (1).

‡ Standard deviation of the mean.

§ The mean value for arginine in chronic granulocytic leukemia (55 ± 6.6) was significantly lower than the mean value in chronic lymphocytic leukemia (85 ± 4.6).

‖ Less than 50 in the other patient analyses.

highly significant, with a probability of correlation equal to 0.999. Patient J.W. (assay G13), in whom the ornithine concentration was normal but the urea concentration very high, had renal insufficiency and therefore was not necessarily producing large amounts of urea. Plasma urea levels of this patient were 26,000 μmoles per kg water compared with a normal value of 5,380; the excretion rate of urea was 3,800 μmoles per hour compared with a normal excretion of 11,450.

Ethanolamine was found in eight specimens of plasma from eight patients; it was never found in any of the analyses of normal subjects. β-Amino isobutyric acid was found in two analyses (G13 and L1). This substance also was not found in the plasma of normal subjects and we know of no reports of its being found by others. The only substance in low concentration in the plasma of the patients was alanine.

Arginine concentrations were observed to be low in the plasma of chronic granulocytic leukemic patients by Kelley and Waisman (2). Our determinations of arginine in such patients were somewhat lower, but not significantly lower than the arginine values found in normal subjects (1). In chronic lymphocytic leukemia the arginine concentrations tended to be slightly higher than those found in normal subjects. Comparison of the means of the arginine values of these two groups of patients showed their differences to be significant.

Kelley and Waisman also reported significantly higher values for proline and phenylalanine in leukemic patients. The mean concentrations for these two substances in our patients were moderately higher but not significantly higher than those found in normal subjects. High concentrations of glutamic acid and alanine in chronic leukemic patients or low concentrations of threonine and high concentrations of tyrosine in acute leukemic patients, which had been observed by Kelley and Waisman (2), were not found in the current study.

Unbound amino acids in erythrocytes of leukemic patients

The concentrations of unbound amino acids found in the erythrocytes are presented in Table II (assays G9 and L18 were lost). The erythrocyte-plasma ratios are presented in Table III (assay G9, M2 and L18 not included). As with the plasma analyses, values less than, or greater than, 2 SD from the normal mean are listed in the fourth and fifth columns of these tables. With the exception of ergothioneine (discussed below), there were no patterns in amino acid concentrations that could be related to the types of leukemia or to the treatment of the patients. The total unbound amino acid concentrations were approximately 10 per cent higher in the erythrocytes of the patients than in normal subjects. Most of this increase was attributable to glutamic acid and serine plus glycine which were 50 per cent and 40 per cent higher, respectively. Four other substances, ornithine, proline, ethanolamine and urea were also significantly higher. On the other hand the erythrocyte-plasma ratios of only alanine and serine plus glycine were higher than those found in normal subjects. The glutamic acid ratio could not be calculated in view of the absence of this substance in plasma. Ornithine and urea were therefore higher in both the plasma and erythrocytes of the leukemic patients. The same was probably true for proline; its mean concentration was elevated in both the plasma and erythrocytes but only in the erythrocytes was it elevated significantly above the normal mean. The high erythrocyte-plasma ratio for alanine was attributable to the low plasma values for this substance. Serine plus glycine was high in the erythrocytes but showed no change in plasma concentration. The high values of taurine found in the erythrocytes are undoubtedly due to a greater leukocyte contamination in the patients' erythro-

TABLE II

Concentrations of unbound amino acids in erythrocytes of leukemic patients

	No. of analyses	Mean	pᴍ*	Analyses with values 2SD less than normal mean†	Analyses with values 2SD higher than normal mean†
		μmoles/kg water			
Alanine	32	419 ± 24‡	ns	M2	G7, 10, 11, 12, L8
α-Amino-*n*-butyric acid	23	30 ± 3	ns	L13, 14	G4, L2, 3, 4, 8, 10a
Arginine	26	<16	ID§	0	G1, L2
Glutamic acid	32	564 ± 50	SH (0.03)	G3	G1, 5, 13, M2, L1, 3, 7, 8, 12, 13, 15, 16
Glutamine	32	543 ± 25	ns	0	0
Histidine	28	130 ± 9	ns	G2, M1, L13	G12, 13, L8, 10a
Lysine	32	250 ± 15	ns	0	G1, L3
Methionine	21	32 ± 2	ns	M2	G1, 3, L8, 16
Ornithine	32	232 ± 13	SH (0.30)	M1	G6, 7, 12, L3, 7–9, 16, 17
Phenylalanine	31	70 ± 9	ns	G2	L7, 8, 9, 16
Proline	32	229 ± 13	SH (0.40)	0	G7, 13, M1, L3, 8
Threonine	31	180 ± 10	ns	L10, 14	G4, 7, 11, 13, L8, 15, 16
Tryptophan	30	22 ± 2	ns	G2, 4, L10a	0
Tyrosine	32	76 ± 5	ns	G2, 5, M1	L4, 16
Valine	32	228 ± 12	ns	G12	G1, 7, 13
Ergothioneine‖	21	439 ± 62	ns	L14	L5, 6, 7
Ergothioneine¶	8	118 ± 13	SL (0.05)	G3	0
Ethanolamine	31	<23	H	0	G1, 2, 4, 7, 8, 13, L3, 8, 9, 13, 15
Leucine plus isoleucine	31	234 ± 16	ns	0	G1, 7, L8
Serine plus glycine	31	1,316 ± 81	SH (0.20)	0	G4, 5, 7, 12, 13, L2, 3, 5, 8, 10a, 12, 15–17
Taurine	31	<245 ± 110	H	0	G1, 4, 7, 10–13, L3, 12, 13, 15–17
Urea	29	8,728 ± 890	SH (0.30)	0	G13, L2, 5–9, 12, 16, 17

* See footnote to Table I.
† Taken from reference (1).
‡ Standard deviation of the mean.
§ Indeterminate.
‖ Ergothioneine analyses from all patients except those with chronic granulocytic leukemia.
¶ Ergothioneine analyses from chronic granulocytic leukemic patients.

TABLE III

Erythrocyte-plasma ratio of unbound amino acids in leukemic patients

	No. of analyses	Mean	PM*	Analyses with values 2SD less than normal mean†	Analyses with values 2SD higher than normal mean†
		μmoles/kg water			
Alanine	31	1.32 ± .05‡	SH (0.13)	0	G1, 2, 3, 7, 12, 13, M1, L8, 9, 10a, 13, 16
α-Amino-*n*-butyric acid	19	1.31 ± .16	ns	L14	L2, 8, 10a
Glutamine	30	1.09 ± .06	ns	0	0
Histidine	26	1.43 ± .09	ns	0	L8
Lysine	30	1.19 ± .08	ns	0	G1, 3
Methionine	20	1.68 ± .15	ns	0	0
Ornithine	28	2.15 ± .14	ns	G10	0
Phenylalanine	29	1.10 ± .05	ns	0	0
Proline	31	1.18 ± .05	ns	0	L8, 12
Threonine	29	1.17 ± .07	ns	0	G4, 7, L8, 11
Tyrosine	31	1.12 ± .06	ns	0	G12, L8, 16
Valine	31	1.24 ± .06	ns	0	G1, 12, L8, 12, 15
Ethanolamine	8	1.48 ± .25	ID§		
Leucine plus isoleucine	30	1.18 ± .07	ns	G6	G12, L8
Serine plus glycine	29	2.14 ± .16	SH (0.10)	G10	G3, 5, 7, 12, 13, L5, 10a, 14
Urea	28	1.13 ± .05	ns	M1	G2, L10a, 12

* See footnote to Table I.
† Taken from reference (1).
‡ Standard deviation of the mean.
§ Indeterminate.

cytes. The very high levels of glutamic acid found in the erythrocytes will be further discussed below.

Iyer (5) has reported marked increases in concentrations of alanine, glutamic acid, glutamine, and serine plus glycine in red cells of patients with iron deficiency anemia. There was no evidence that any of our patients had iron deficiency anemia but several of them had moderate to severe anemia associated with their leukemia. The specimens of anemic patients showed no consistent variation from the specimens of patients with no anemia with regard to any of the amino acids.

The concentrations of ergothioneine found in the erythrocytes of the patients with chronic granulocytic leukemia were much lower than those found in other leukemic patients or in normal subjects. Since almost nothing is known of ergothioneine function in mammalian cells the significance of this striking difference is not clear.

Abnormal concentrations of leukocyte amino acids which were common to most of the leukemic patients

Changes in concentration of four substances in the leukocytes of the patients, when compared with normal subjects, were characteristic of leukemia without regard to the kind of leukemia and, except for treatment with Chlorambucil,[1] were independent of the type of treatment (see Tables IV, V, VI and VII). These were O-phosphoethanolamine, glutamic acid, proline and ornithine. Discussions of each of these follow.

O-phosphoethanolamine concentrations were found to be very high in the leukocytes of most leukemic patients; values higher than the normal means were found in every patient sample in which O-phosphoethanolamine was measured. All untreated chronic lymphocytic leukemic patients, acute granulocytic leukemic patients, and monocytic leukemic patients had O-phosphoethanolamine concentrations which were higher by more than 2 SD of the normal mean. It was also high in patients after treatment with X-ray, TEM [2] and nitrogen mustard.[3] Its concentration tended to be less high in chronic granulocytic patients; only one patient in six (G1) had values higher than 2 SD of the normal mean. O-phosphoethanolamine was not determined in the leukocytes of Patients G3, 4, 7 and 8. Its concentration was normal in two cases after treatment with Chlorambucil. Elevated concentrations of O-phosphoetha-

[1] 4-[p-*bis*(2-chloroethyl)aminophenyl]butyric acid.
[2] 2,4,6-Triethyleinimino-s-triazine.
[3] 2,2′-Dichloro N-methyldiethylamine.

nolamine had previously been observed by Nour-Eldin and Wilkinson in the leukocytes of some leukemic patients (3). In addition to this, Outhouse (6) as early as 1936, had found increased amounts of O-phosphoethanolamine in many malignant tissues. It is also highly significant that other patients, such as those with leukemoid reaction, polycythemia vera, leukocytosis following accidental cobalt-radiation, malignant carcinoid, congenital anemia, spherocytosis and general emaciation did not show elevated O-phosphoethanolamine levels.[4]

Glutamic acid concentrations had been found to be elevated in the leukocytes of leukemic patients by Nour-Eldin and Wilkinson (3). In the present study the mean concentration for glutamic acid in the granulocytic, monocytic and untreated chronic lymphocytic leukemic patients was more than twice as high as the mean concentration found in normal subjects. In 13 of the 24 patients in these categories glutamic acid analyses were higher than the normal mean by more than 2 SD. Leukocyte glutamic acid concentrations were also very high in two analyses conducted on a patient one and two days after extensive irradiation. On the other hand, only one of the six patients under treatment with chemotherapeutic agents had an elevated glutamic acid value. The high glutamic acid values in the leukocytes in conjunction with the high concentrations in the erythrocytes of these leukemic patients emphasizes that the metabolism of this amino acid is critically affected in leukemia. High glutamic acid values have also been noted in other malignant tissues (7, 8). It was not found to be characteristically elevated in either the erythrocytes or leukocytes of the nonleukemic patients listed above.

Proline concentrations also were high in the leukocytes of the leukemic patients, particularly when compared with the concentrations of the other amino acids. It was higher than 2 SD of the normal mean in five patients with granulocytic leukemia (category *a*, see below); it was low in concentration in only one patient, that of assay L3, where the unbound amino acid concentrations were almost all very low. Proline concentrations were also elevated in the erythrocytes of the leukemic patients (Table II). Since glutamic acid and proline have interrelated pathways of metabolism (9), a correlation study by regression analysis was conducted. This study did not, however, support a relationship between these two substances in either the erythrocytes or leukocytes.

Low ornithine concentrations were observed in the leukocytes of many of the leukemic patients. Ornithine was elevated in assays of only two patients, L6 and L8. However, in both of these analyses the other amino acid concentrations were increased severalfold. Ornithine concentrations were less than 2 SD of the normal mean in 15 analyses out of 29. This low level of ornithine in the leukocytes becomes even more striking in view of the higher than normal concentrations of ornithine found in the plasma and erythrocytes of these same patients.

Arginine concentrations in the leukocytes of the patients were variable. In most instances no arginine was found. However, in a few patients (G1, G11, L1, L6) high concentrations of arginine were found (roughly 1,000 μmoles per kg water). No significance can be attributed to this finding, since similar irregular arginine values had been found in two of the leukocyte analyses of normal subjects (1).

Abnormal concentrations of leukocyte amino acids which depended upon the type of leukemia or its treatment

The concentration of the amino acids in the leukocytes of the leukemic patients, other than the four amino acids discussed above, showed considerable variation. In some instances the variation could be correlated with the type of leukemia and its treatment. For the purpose of this evaluation the patients have been grouped into four categories: *a*) granulocytic and monocytic leukemia, *b*) untreated chronic lymphocytic leukemia (untreated patients were classified as those who had had no chemotherapy or X-ray irradiation, or had not had such treatment for a period of four months preceding the assay),[5] *c*) chronic lympho-

[4] Unpublished studies.

[5] There were also included in category *b* patients who had been started on treatment with Chlorambucil within eight days before the assay. Treatment with Chlorambucil usually does not result in immediate changes as indicated by white count depression. Furthermore, in every instance the analyses from patients in this early stage of treatment were similar to the untreated cases.

TABLE IV

Concentration of unbound amino acids in leukocytes of granulocytic and monocytic leukemic patients *

	No. of analyses	Mean	pm†	Analyses with values 2SD less than normal mean‡	Analyses with values 2SD higher than the normal mean‡
		μmoles/kg water			
Alanine	13	3,351 ± 553§	ns	0	G7
Arginine	11	<368 ± [99]	ns	0	0
Glutamic acid	13	6,048 ± 875	SH (0.3)	0	G1, 5, 6, 7, 11, M1, 2
Glutamine	13	2,844 ± 405	ns	G9	G1
Histidine	10	546 ± 110	ns	G9, 13, M2	0
Lysine	13	1,947 ± 214	ns	0	0
Methionine	10	<450 ± [51]	ns	0	0
Ornithine	10	<1,310 ± [141]	SL (0.25)	G4, 7, 9,	0
Phenylalanine	13	683 ± 73	ns	0	0
Proline	13	1,378 ± 127	SH (0.10)	0	G1, 3, 7, 13, M1
Threonine	11	1,788 ± 214	ns	G9	0
Tryptophan	13	171 ± 13	ns	0	0
Tyrosine	13	565 ± 75	ns	0	0
Valine	13	1,366 ± 130	ns	G9	0
Ethanolamine	13	<286 ± [54]	ns	0	0
Leucine plus isoleucine	13	2,494 ± 322	ns	0	G3, 11
Serine plus glycine	12	9,819 ± 1,650	ns	0	0
Taurine	13	37,211 ± 3,730	ns	0	G1, 11, M1
O-phosphoethanolamine	10	10,263 ± 2,610	SH (0.50)	0	G1, 5, 6, M1, 2
Cell volume‖	12	0.569 ± 0.050	ns	G13	0

* Patient assays G1–11, 13, M1, 2.
† See footnote* to Table I.
‡ Taken from reference (1).
§ Standard deviation of the mean. Values in brackets are approximate.
‖ Volume of one billion leukocytes.

cytic leukemia treated within the four month period preceding the assay by chemotherapeutic agents, and *d*) chronic lymphocytic leukemia treated by radiation one or two days before the assay. Tables IV, V, VI and VII present the results obtained in the study of these various categories.

a) *Granulocytic and monocytic leukemia.* The results of the determinations of the unbound amino acids in the leukocytes of the granulocytic and monocytic leukemic patients are given in Table IV. High concentrations of glutamic acid, O-phosphoethanolamine and proline, and a low concentration of ornithine, all of which have been already found in most leukemic patients, were the only differences from normal subjects noted in these analyses. Furthermore, when examined with respect to the types of leukemia grouped in Table IV (chronic granulocytic leukemia, acute granulocytic leukemia, subacute granulocytic leukemia and monocytic leukemia) there were no patterns that could be associated with any one type of leukemia. Treatment of the patients by Myleran, 6-mercaptopurine, Meticorten, testosterone or spray X-ray of the spleen five days before the analysis caused no recognizable changes in amino acid concentrations in the leukocytes of this group of patients.

The packed-cell volumes of the leukocytes of the granulocytic and monocytic patients were found to be similar to those of normal subjects. Platelet contamination occurred to an appreciable extent in analyses G2 and G3 (25 platelets to approximately 1 leukocyte). Comparison of the amino acid concentrations found in platelets [6] with those in leukocytes showed that this contamination would not have affected the leukocyte values by more than 15 per cent and no allowance was made for it. One would expect the error induced by platelet contamination to be largely compensated for in the packed-cell volume measurements which were used to express the concentration of the amino acids and which included the volumes of both the platelets and the leukocytes.

b) *Untreated chronic lymphocytic leukemia.* Table V presents the results of 11 analyses of leukocytes from 10 patients with chronic lympho-

[6] Unpublished experiments.

cytic leukemia who had not had chemotherapy or X-ray treatments, or who had not had such treatment for a period of four months preceding the assay. Only alanine, proline, tyrosine and probably arginine were at concentrations comparable with those in the leukocytes of normal subjects. Glutamic acid, O-phosphoethanolamine and ethanolamine were at very high concentrations. Excluding these seven substances the average concentrations of the remaining amino acids were 50 per cent less than those of normal subjects. Glutamine concentration was only 40 per cent of that found in normal leukocytes. This substance was also found to be slightly lower in the erythrocytes of the leukemic patients, but it was not lower in the leukocytes of granulocytic and monocytic leukemias (category *a*) Rouser (4) and others (8) noted a tendency for low glutamine values in malignant tissues and have discussed its possible significance.

A serious drawback in the current study of patients with lymphocytic leukemia was the failure to obtain analyses of pure lymphocytes from normal subjects. The lymphocytes comprise not more than 15 per cent of the total packed-cell volume in normal blood analyses. We have therefore no accurate estimate of the unbound amino acid concentrations in normal lymphocytes and could only compare the concentration of the amino acids in the lymphocytes of these patients with normal leukocytes which include different cell types. In the study of the concentrations of unbound amino acids in treated lymphocytic leukemic patients, to be discussed in the following sections, large changes were observed, which depended upon the patients' treatment. For this reason it was very helpful to know the concentrations of the amino acids in the leukocytes of untreated patients.

c) Chronic lymphocytic leukemia after treatment with chemotherapeutic agents. In this category are included three patients who had been treated with 4-[p-*bis*(2-chloroethyl)aminophenyl] butyric acid (Chlorambucil, Burroughs Wellcome; two analyses were conducted on one patient after two different periods of treatment); one patient who had been treated with 2,4,6-triethyleneimino-s-triazine (TEM, Lederle); and one patient who had been treated with nitrogen mustard (2,2'-

TABLE V

Concentration of unbound amino acids in leukocytes of untreated chronic lymphocytic leukemic patients *

	No. of analyses	Mean	pu†	Analyses with values 2SD less than normal mean‡	Analyses with values 2SD higher than normal mean‡
		μmoles/kg water			
Alanine	11	2,739 ± 257§	ns	0	0
Arginine	10	<151 ± [23]	ns	0	0
Glutamic acid	11	5,236 ± 640	SH (0.45)	0	L2, 5, 9, 10a, 12, 13
Glutamine	11	1,092 ± 97	SL (0.05)	L10, 12, 15	0
Histidine	11	<360 ± [53]	SL (0.10)	L9, 10, 10a, 12, 15	0
Lysine	10	977 ± 112	SL (0.10)	L10, 12	0
Methionine	8	<188 ± [21]	SL (0.30)	0	0
Ornithine	10	1,169 ± 146	SL (0.20)	L5, 9, 10, 11, 12, 15	0
Phenylalanine	10	<312 ± [20]	SL (0.20)	0	0
Proline	11	670 ± 65	ns	0	0
Threonine	10	1,166 ± 148	SL (0.05)	L10, 12, 13, 15, 18	0
Tryptophan	11	<126 ± [20]	SL (0.50)	L10, 15	0
Tyrosine	11	<313 ± [44]	ns	0	0
Valine	11	709 ± 109	SL (0.30)	L12, 15	0
Ethanolamine	11	<431 ± [129]	high	0	L9, 11
Leucine plus isoleucine	9	<896 ± [148]	SL (0.10)	L12, 15, 16	0
Serine plus glycine	11	7,235 ± 790	SL (0.40)	0	0
Taurine	11	15,233 ± 1,410	SL (0.20)	0	0
O-phosphoethanolamine	11	9,282 ± 1,070	SH (0.02)	0	L2, 5, 9, 10, 10a, 11, 12, 13, 15, 16, 18
Cell volume‖	11	0.247 ± 0.012	SL (<0.01)	all low	0

* Patient assays L2, 5, 9, 10, 10a, 11, 12, 13, 15, 16, 18.
† See footnote* to Table I.
‡ Taken from reference (1).
§ Standard deviation of the mean. Values in brackets are approximate.
‖ Volume of one billion leukocytes.

TABLE VI

Concentration of unbound amino acids in leukocytes of treated chronic lymphocytic leukemic patients *

	Chlorambucil				TEM	Nitrogen mustard
	L1	L6	L3	L4	L14	L17
Alanine	2,030	7,200 ↑	330 ↓	3,550	1,240	2,690
Arginine	1,000	800	<100	200	<100	100
Glutamic acid	3,100	4,230	750 ↓	3,900	5,200 ↑	4,290
Glutamine	2,200	2,530	1,500	1,100	1,100	2,550
Histidine	<200 ↓	3,660 ↑	<200 ↓	90 ↓	620	950
Lysine	2,900	5,950 ↑	<500	610	410 ↓	1,680
Methionine	660		<100			310
Ornithine	<400 ↓	16,000 ↑	<200 ↓	<200 ↓	330 ↓	700 ↓
Phenylalanine	700	<600	150	<300	950	2,200
Proline	730	2,800 ↑	<200 ↓	530	870	1,630 ↑
Threonine	<700 ↓	6,400 ↑	970 ↓	735 ↓	1,040	1,570
Tryptophan	290	600 ↑	<50	60	150	550
Tyrosine	880	2,700 ↑	180	210	400	900
Valine	1,460	2,800 ↑	430	430	820	1,230
Ethanolamine	<300	<600	1,950 ↑	<300	<200	<200
Leucine plus isoleucine		6,750 ↑	330 ↓	590 ↓	1,000	1,290
Serine plus glycine	8,400	71,000 ↑	2,700 ↓	4,300	87,000 ↑	9,000
Taurine	21,000	49,000	10,600	19,700	22,000	13,600
O-phosphoethanolamine		3,400		4,700	5,580 ↑	12,400 ↑
Cell volume†	0.37 ↓	0.31 ↓	0.31 ↓	0.27 ↓	0.33 ↓	0.28 ↓
Cell count‡	9.3	5.0	67.0	56.0	9.3	57.0

* The values are expressed as micromoles per kilogram water. The symbol ↓ following the number in the column indicates that the value is at least 2 SD below the normal mean. The symbol ↑ following the number indicates that the value is at least 2 SD above the normal mean (1).

† Volume of one billion leukocytes.

‡ Counts per $mm^3 \times 10^{-3}$ of whole blood.

dichloro-N-methyldiethylamine, Merck). The assays of these patients are listed in Table VI. As previously noted, the high concentrations of O-phosphoethanolamine typical of the untreated chronic lymphocytic leukemias were not uniformly found in this group of patients. Furthermore, glutamic acid was not as high in the leukocytes of this group as in other leukemic patients. Other amino acids showed large variations in concentration which appeared to be influenced by the type of treatment and period of time after treatment. A discussion of these effects in each of these patients follows.

In the leukocytes of two patients (assays L1 and L6) shortly after treatment with Chlorambucil, concentrations of amino acids were considerably higher than those found in untreated patients. Thus, in assay L1, from Patient H.B. on the tenth day after the start of a three week course of Chlorambucil, amino acid concentrations were approximately 40 per cent higher than those of untreated chronic lymphocytic patients. With the exception of histidine, ornithine, and threonine, whose concentrations were low, the unbound amino acid concentrations for this assay were comparable with the concentrations in leukocytes of normal subjects. In analysis L6, taken from Patient A.J.R. on the nineteenth day after completion of a three week course of Chloroambucil, a very marked increase in the unbound amino acid concentrations was observed. The values were much higher than the concentrations which had been found for untreated chronic lymphocytic leukemics and were higher than 2 SD from the means from normal leukocytes in all substances except for glutamic acid, glutamine, taurine and O-phosphoethanolamine. The white cell counts of both of these patients had been brought down by the treatment from high to normal levels. The volume of the cells was unchanged by the chemotherapy. The differential count for Patient H.B. showed 87 per cent lymphocytes and 9 per cent polymorphonuclear neutrophils. The differential count for Patient A.J.R. in analysis L6, however, had been changed from predominantly lymphocytes before treatment to a differential count of 70 per cent polymorphonuclear neutrophils, 10 per cent lymphocytes and 6 per cent myelocytes after treatment. The large change in unbound amino acid concentrations therefore accompanied a

change in the proportion of cell types in this patient. This increase could not be explained solely by a change in cell type, since other patients with cell types similar to those in assay L6 did not show elevated amino acid concentrations.

Two analyses from one patient were taken 44 days (assay L3) and 75 days (assay L4) after completion of different series of Chloroambucil treatment (see Table VI). The unbound amino acid concentrations found in each were either as low as or lower than the concentrations found in untreated patients. Presumably the critical stage of elevated amino acid concentrations following treatment had passed. However, the response of this patient to Chlorambucil had not been satisfactory as far as white count depression was concerned. Analysis L3 was unique in that it showed the lowest unbound amino acid concentration ever found. The average concentration was approximately one-fourth that found in normal leukocytes. Glutamine was normal and ethanolamine was elevated. The unbound amino acid analyses of the urine for this assay were also very low. The white count was 67,000 per mm^3 for analysis L3 and 56,000 per mm^3 for analysis L4. The cell type in both analyses was essentially all lymphocytic.

One assay (L14) was conducted on a patient on the twelfth day following completion of treatment with TEM, and another assay (L17) was conducted on a patient who had had treatment with nitrogen mustard on the eighteenth, seventeenth and fourth days prior to the assay. The results of these assays are listed in Table VI. O-phosphoethanolamine was elevated in the leukocytes of both of these patients, glutamic acid was elevated in one. Other amino acid concentrations were irregular but usually higher than those found in untreated chronic lymphocytic leukemic patients. A different response to treatment by Chlorambucil and these two compounds in relation to O-phosphoethanolamine and glutamic acid reduction is thus implied although the number of subjects involved is very small.

d) Chronic lymphocytic leukemia after X-ray treatment. A patient with chronic lymphocytic leukemia and large deposits of lymphosarcoma had two tests conducted in association with X-ray treatment and a third test conducted well after the period of X-ray treatment; Table VII lists the leukocyte and urine values for these analyses. The first analysis was made on the day after the first exposure to 20 r units of total body radiation. Alanine, glutamic acid, taurine and O-phosphoethanolamine concentrations in the leukocytes were elevated 5-, 20-, 4- and 20-fold, respectively, above the mean of normal subjects as well as above that of the untreated lymphocytic leukemic patients. Several of the other amino acids were moderately elevated, whereas only histidine and ornithine were low. After completion of the first course of X-ray treatment, the white cell counts of the patient were reduced from 487,000 to 9,500 per mm^3 in three months, but the masses of lymphosarcoma in various areas of the lymph nodes were not much reduced. A second course of X-ray consisting of 40 r units per treatment to large areas of the body, but not total body radiation, was given on the sixth, fourth, third and second days prior to the second analysis. Some reduction in the quantity of the amino acids in very high concentrations was noted when compared with the earlier analysis. However, alanine, glutamic acid, leucine plus isoleucine, lysine, threonine, and serine plus glycine were elevated approximately twofold above the normal range and O-phosphoethanolamine was elevated eightfold. On the third analysis, 135 days following the second course of X-ray treatment, and with no intervening treatment of any kind except transfusions, the leukocyte levels had returned to the state of the typical untreated chronic lymphocytic leukemia. The elevated amino acid concentrations in the leukocytes following X-ray treatment were not influenced by the magnitude of the white cell count as these differed enormously: 490,000, 9,500 and 22,000 per mm^3 for bloods from analyses L7, L8 and L9, respectively. No changes occurred in cell type following X-ray (all lymphocytes). The packed-cell volumes of the leukocytes in these assays were not significantly larger than the packed-cell volumes of lymphocytic leukemic patients who had not had X-ray irradiation. This does not agree with the increases in cell volumes observed by others following *in vivo* irradiation of Yoshida sarcoma cells in rats (10) or Ehrlich carcinoma cells in mice (11).

Except for an increased concentration of urea

TABLE VII

Relationship between the unbound amino acid concentrations in the leukocytes and their excretion rates in the urine of a patient following X-ray treatments *

	L7 24 hrs after X-ray		L8 48 hrs after X-ray		L9 135 days after X-ray	
	Leukocytes	Urine	Leukocytes	Urine	Leukocytes	Urine
Alanine	13,500 ↑	11.0	8,500 ↑	14.6	3,760	10.0
Arginine			100		290	
Glutamic acid	65,000 ↑		8,500 ↑		6,570 ↑	
Glutamine	7,100 ↑	66.0 ↑	2,580	28.6	1,570	15.0
Histidine	200 ↓	40.0	1,600 ↑	68.0	240 ↓	35.0
Lysine	5,450 ↑		5,900 ↑	12.7	1,410	3.7
Methionine	500		100			
Ornithine	500 ↓		2,850		940	
Phenylalanine	930	5.0	1,510 ↑	8.3	300	2.1
Proline	1,440		2,000 ↑		970	
Threonine	4,850 ↑	16.6	5,830 ↑	11.0	1,290	9.0
Tryptophan	250		460		100	2.2
Tyrosine	1,000	6.4	1,050 ↑	4.1	450	2.8
Valine	1,600	4.8	3,200 ↑	3.6	940	1.0
Ethanolamine		68.0 ↑	840 ↑	17.0	890 ↑	2.7
Leucine plus isoleucine	2,400		5,250 ↑		1,100	
Serine plus glycine	20,200	56.0	46,000 ↑	97.0	8,900	35.0
Taurine	115,000 ↑	220.0 ↑	21,600	116.0 ↑	23,600	28.0
Urea		21,400		15,400		11,300
O-phosphoethanolamine	63,000 ↑		23,000 ↑		6,400 ↑	
Cell volume†	0.31 ↓		0.31 ↓		0.28 ↓	
Cell count‡	490.0		9.5		22.0	

* Leukocyte values are expressed as micromoles per kilogram water. The urine excretion rates are expressed as micromoles excreted per hour. The symbol ↓ following a leukocyte value indicates that it is at least 2 SD below the normal mean; the symbol ↑ following a value indicates that it is at least 2 SD above the normal mean (1). For the urines, these symbols indicate that the values are 50 per cent less than the lowest normal value or 200 per cent greater than the highest normal value (5).

† Volume of one billion leukocytes.

‡ Counts per $mm^3 \times 10^{-3}$ of whole blood.

in the plasma and erythrocytes, and an increased concentration of glutamic acid in the erythrocytes, irradiation did not affect the unbound amino acids in the blood compartments other than the leukocytes.

High concentrations of phenylalanine and ornithine were observed in the plasma and erythrocytes of this patient (S.G.) without regard to the time the assay was conducted after irradiation, and therefore these elevated values do not appear to be associated with radiation exposure.

Katz and Hasterlik (12) found, in a study of individuals accidentally exposed to varying amounts of total body irradiation, that while aminoaciduria was evident 12 hours after exposure the peak excretion was not reached until the sixth day. Kay, Early and Entenman (13), however, had observed a peak excretion of taurine and urea in rats five hours after irradiation. In the curent study, in which exposure to irradiation had occurred approximately 24 hours prior to assay L7 and approximately 48 hours prior to assay L8, taurine excretion rates were very high in both assays, and urea, glutamine, and ethanolamine excretion rates were moderately high. Of considerable interest in this study was the correlation between the high excretion rates and high concentrations of some compounds in the plasma, erythrocytes and leukocytes following the X-ray exposure. Thus the high excretion rate of taurine corresponded to high concentrations of taurine in the leukocytes. High excretion rates of glutamine corresponded to high concentrations of glutamic acid (the precursor for the glutamine excreted in the urine) in the leukocytes and erythrocytes. High excretion rates of ethanolamine in assay L7 corresponded to a very high concentration of O-phosphoethanolamine in the leukocytes (O-phosphoethanolamine is presumably hydrolyzed to give ethanolamine). Finally, high excretions of urea corresponded to high concentrations of urea in the plasma and erythrocytes.

Excretion of amino acids in the urines of leukemic patients

The means and ranges of the excretion rates for 29 of the leukemic patients are given in Table VIII. Assays L7 and L8 were omitted from this table since some of their values were very high and they have been presented separately in Table VII; assays L12 and L15 were lost. Columns 5 and 6 of this table list the patients with very low or very high excretions for particular substances. The mean excretion rates of the patients are reasonably close to those of normal subjects. Several substances showed occasional large fluctuations in the patients' excretion. Taurine was high in two analyses, L4 at 80 μmoles per hour and M2 at 310 μmoles per hour. The patient with assay L4 was on Day 75 following a course of Chlorambucil and on the third day after the start of a new course of Chlorambucil, but was not otherwise unusual. The patient with assay M2, in addition to extremely high taurine excretion also had a moderately high excretion rate for many other substances. This patient, in addition to monocytic leukemia, had an acute rectal abscess which may have influenced the amino acid excretion pattern. Several of the patients had low taurine excretion rates which did not appear to be associated with any particular form of leukemia or treatment. In the analysis of one patient (assay L10a) serine plus glycine was excreted at the very high level of 390 μmoles per hour with somewhat moderate increases in excretion of the other amino acids. An unusual feature of this patient was that in an analysis 74 days earlier, and with no apparent intervening change having taken place in the patient's condition, a normal excretion rate for all substances was found (assay L10). Proline and valine, which were usually not found in the urines of normal subjects, were excreted in increased amounts by some of the patients.

A rather unusual finding was the low excretion rate for ethanolamine in six urines of patients with chronic lymphocytic leukemia. Ethanolamine was elevated in the plasma and erythrocytes of some of the patients. However, except for an analysis following X-ray exposure, ethanolamine was never excreted by the patients in amounts greater than those excreted by normal subjects. This suggests that ethanolamine is being utilized in some manner, perhaps associated with O-phosphoethanolamine formation, rather than being excreted. The possibility of the excretion of etha-

TABLE VIII

Rate of excretion of unbound amino acids in urines of leukemic patients *

	No. of analyses	Mean	Range	Analyses with values less than 50% of lowest normal value†	Analyses with values higher than 200% of highest normal value†
Alanine	29	7.8	1.8–18.5	0	0
Arginine	11	<0.5		0	0
Glutamine	27	13.8	1.8–30.0	G13, L3	0
Histidine	29	28.1	5.0–79.0	0	0
Lysine	26	7.0	2.1–25.0	0	0
Methionine	29	<1.0		0	0
Ornithine	19	<2.0		0	0
Phenylalanine	29	<1.6	<1.0– 4.4	0	0
Proline	29	<1.0	<0.3– 3.9	0	G4, 8, L3
Threonine	26	5.7	2.1–13.0	0	0
Tryptophan	29	<1.9	<0.3– 5.4	0	0
Tyrosine	29	<2.9	<1.0– 8.0	G13	0
Valine	29	<1.8	<1.0– 8.3	0	M2, L4, 10a, 14
Ethanolamine	27	<12.9	<1.0–44.0	L1, 6, 9, 13, 16, 17	0
Guanidoacetic acid	11	<10.2	<1.0–24.0	G13	0
Leucine plus isoleucine	29	<2.3	<1.0– 4.0	0	0
Serine plus glycine	29	55.0	<8.0–390.0	G13	L10a
Taurine	29	31.5	2.7–310.0	G4, L1, 10, 16	M2, L4
Urea	28	10,000	2,900–19,400	0	0
β-Amino isobutyric acid	2	17.0	14 20		L1, 3

* Urines of assays L7, L8, L12, and L15 are excluded from these averages. Values are expressed as micromoles excreted per hour.

† The ranges for the normal values are taken from reference (5).

nolamine in a complex form was not investigated. In any event, the results of this study strongly support a disturbed ethanolamine and O-phosphoethanolamine metabolism in the leukemic patients.

β-Amino isobutyric acid, which was found infrequently in our analyses of normal subjects (1), was found in moderate amounts in urine assays L1 and L3; both patients had had treatment with Chlorambucil. In some of our assays an unidentified ninhydrin-positive substance in very high concentration interfered with the estimation of β-amino isobutyric acid. Fink, Henderson and Fink (14) first reported the presence of β-amino isobutyric acid in urine. These workers noted that its concentration was occasionally very high in cancer patients and apparently related to the neoplastic process. Awapara (15) found β-amino isobutyric acid to be excreted in large amounts in the urines of leukemic patients treated with nitrogen mustard, provided the analysis coincided with a particular time interval after the treatment.

DISCUSSION

In all four of the body compartments of leukemic patients (that is, plasma, erythrocytes, leukocytes, and urine) differences from normal subjects have been found to exist. These are reported in some detail in the preceding section. For brevity and easy reference the principal findings in each compartment are also tabulated in Table IX. It is important to recognize that while some changes were reflected simultaneously in two compartments, e.g., increase in glutamic acid and proline in both erythrocytes and leukocytes of the leukemic patients, other changes occurred in only one specific compartment, e.g., increase in serine plus glycine in the erythrocytes, increases of ornithine in the erythrocytes, or decreases of ornithine in the leukocytes. By virtue of these different responses, it must be concluded that in leukemia each of the fluid compartments is affected in a different manner.

One may speculate at some length on the significance of the complex changes observed in the leukemic patients. One of the most striking, and certainly one which appears to be common to the neoplastic process, since it is also found in other malignancies (7, 8), is the elevated cell glutamic acid concentration. In the present study high glutamic acid was observed not only in the leukocytes but also in the erythrocytes. The cause of its elevation is not clear; it may be due to an alteration in metabolism of the cancer cells, such as in the citric acid cycle where α-ketoglutarate

TABLE IX

Tabulation of the principal differences in concentrations of unbound amino acids and other substances found in the study of the leukemic patients when compared with normal subjects *

	Plasma	Erythrocytes	Leukocytes				Urine†	
			G&ML	UCLL	CCLL	XCLL	XCLL	Other leukemic patients
Alanine	SL	ns	ns	ns	fluct	SH	ns	ns
Glutamic acid		SH	SH	SH	fluct	SH		
Glutamine	ns	ns	ns	ns	ns	fluct	high	ns
Ornithine	SH	SH	SL	SL	fluct	fluct		
Proline	ns	SH	SH	ns	fluct	fluct	ns	ns
Ergothioneine (CGL only)		SL						
Ethanolamine	high	high	ns	high	fluct	SH	high	low (UCLL only)
Serine plus glycine	ns	SH	ns	SL	fluct	fluct	ns	fluct
Urea	SH	SH					ns	ns
Taurine			ns	ns	ns	SH	high	fluct
O-phosphoethanolamine			SH	SH	fluct	SH		
Other amino acids	ns	ns	ns	mostly low	fluct	mostly high	ns	ns
Cell volume			ns	SL	SL	SL		

* G&ML = granulocytic and monocytic leukemic patients. UCLL = chronic lymphocytic leukemic patients untreated by chemotherapy or X-ray irradiation. CCLL = chronic lymphocytic leukemic patients treated with chemotherapeutic agents. XCLL = a chronic lymphocytic leukemic patient after X-ray irradiation. CGL = chronic granulocytic leukemia. SH = significantly high. SL = significantly low; ns = not significantly different; fluct = fluctuated.

† The other amino acids include α-amino-*n*-butyric acid, arginine, histidine, lysine, methionine, phenylalanine, threonine, tyrosine, valine, leucine plus isoleucine, serine plus glycine, and ergothioneine (excluding chronic granulocytic leukemia).

is a congener of glutamic acid or in transamidation where glutamic acid is involved, or it may be the result of some unknown defect.

Equally less well understood in its role is the increase of O-phosphoethanolamine in the leukocytes. This substance has also been reported elevated in malignant tissues by others (6). Because it is to a large extent normally fixed in the structure of the cell lipid, the increase may be the result of more rapid turnover of the leukemic as compared with normal cells. The increase in O-phosphoethanolamine concentration in the leukemic white cells is so marked that its use for the diagnosis of leukemia may be feasible. It is of additional interest that both glutamic acid and O-phosphoethanolamine are reduced in concentration in the remission following treatment with Chlorambucil.

A number of changes in concentrations of other substances were noted. Certainly the marked reduction of ergothioneine in the chronic granulocytic leukemic patients should be further investigated. Also, the finding of increased concentrations of amino acids in the leukocytes following extensive body radiation is of much interest. In the latter investigations only small changes were observed in the plasma and erythrocytes, which suggests that the leukocytes may be the more susceptible to radiation damage.

SUMMARY

Interrelationships among the amino acid concentrations in the blood cells, plasma and urine of patients with chronic granulocytic, chronic lymphocytic, acute granulocytic, subacute granulocytic and monocytic leukemia have been studied. When compared with normal subjects the following significant differences were observed.

1. Findings common to all the leukemic patients: *a*) In the plasma, ornithine, ethanolamine and urea were high, alanine was low. *b*) In the erythrocytes, ornithine, proline, ethanolamine, serine plus glycine, and urea were high. *c*) In the leukocytes, O-phosphoethanolamine, glutamic acid and proline were high, ornithine was low. Proline was high in the leukocytes of the chronic lymphocytic leukemic patients only when compared relative to the other amino acids. *d*) Ornithine and urea concentrations were found to be related, that is, increased and decreased together, in the plasma of leukemic patients with adequate kidney function. *e*) Ornithine concentrations increased in the plasma and erythrocytes but decreased in the leukocytes of the leukemic patients.

2. Findings common to certain types of leukemia or treatment of leukemia: *a*) β-Amino isobutyric acid, which was not found in normal plasma, was found in two plasmas after the patients had been treated with Chlorambucil.[1] *b*) Ergothioneine was decreased (average, 75 per cent) in the erythrocytes of chronic granulocytic leukemic patients. *c*) Ethanolamine was high, alanine, proline, tyrosine, and probably arginine were in the normal range; all other substances, except glutamic acid and O-phosphoethanolamine mentioned above, were low in the leukocytes of untreated chronic lymphocytic leukemic patients. *d*) In the leukocytes of two chronic lymphocytic leukemic patients, one 10 days after the start of Chlorambucil treatment, the other 19 days after completion of a series of Chlorambucil treatments, most of the amino acid concentrations were irregularly increased. In the leukocytes of another chronic lymphocytic leukemic patient, on Day 44 and Day 75 following completion of treatment by Chlorambucil, most of the amino acid concentrations were very low. In both of these groups of patients there was a tendency for the concentrations of glutamic acid and O-phosphoethanolamine to decrease from very high values (characteristic of leukemia) to values approaching those of normal subjects. *e*) In the leukocytes of a chronic lymphocytic leukemic patient 12 days after treatment with TEM [2] and a similar patient on Days 18, 17 and 4 after treatment with nitrogen mustard [3] concentrations of amino acids were irregular but somewhat elevated. *f*) In the leukocytes of a chronic lymphocytic leukemic patient 24 hours and 48 hours after X-ray irradiation, most of the amino acids were highly elevated. A high excretion rate of urea, glutamine, taurine and ethanolamine in the urine of this patient correlated well with high concentrations of these substances or their precursors in either the plasma or the blood cells. *g*) In the urine of some of the untreated chronic lymphocytic leukemic patients ethanolamine was excreted in low amounts. Irregular excretion rates of a few other compounds were observed in urines of leukemic patients but without

regard to the type of leukemia. *h*) Arginine was lower in the plasma of chronic granulocytic leukemic patients than in chronic lymphocytic leukemic patients. *i*) Treatment of granulocytic leukemic patients with Myleran (tetra-ethylenemethane sulfonic acid), 6-mercaptopurine, Meticorten, testosterone or spray X-ray to the spleen five days before the analyses caused no recognizable changes in amino acid concentrations in the leukocytes of this group of patients.

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CASE HISTORIES

Terms and abbreviations

The letter and number following the patients' initials are the sex and age. *Prior treatments* are those that were given for leukemia four months or more before the assay. *Recent treatments* are those that were given for leukemia less than four months before the assay. *Hematology.* The leukocyte counts per mm^3 blood (WBC) were taken the day of the assay. Hematocrits (Hct.), grams hemoglobin per 100 ml blood (Hg.), and platelet counts per mm^3 blood (Plat. C.) were taken the day of the assay or several days before.

Aristocort (Lederle) = triamcinolone. Chlorambucil (Burroughs Wellcome) = 4-[p-*bis*(2-chloroethyl)amino phenyl] butyric acid. Meticorten (Schering) = prednisone. Myleran (Burroughs Wellcome) = tetra-ethylenemethane sulfonic acid. Nitrogen mustard (Merck Sharp & Dohme) = 2,2′-dichloro-N-methyldiethylamine hydrochloride. Orinase (Upjohn) 1-butyl-3-ptolysulfonyl-urea. TEM (Lederle) = 2,4,6-triethyleneimino-s-triazine.

Chronic lymphatic leukemia

L1: H.B. M 54 Complication, diabetes mellitus, insulin treatment
Prior treatment, X-ray 1953–1956
Recent treatment, Chlorambucil 6 mg/day 9/21–10/1/57
Red cell transfusion 500 ml 9/30/57
Hematology, WBC 11,600; Hg. 8.3 g Plat. C., 150,000
Assay no. 1 10/1/57

L2: T.McC. M 75 Complication, carcinoma of skin
Prior treatment, none
Recent treatment, Chlorambucil, 10 mg/day 5/21–5/29/58
Hematology, WBC 52,000; Hg. 11.9 g Plat. C., 80,000
Assay no. 1 5/29/58

L3: A.R. F 67 Complication, diabetes mellitus, insulin treatment
Prior treatment, X-ray 1955–1956
TEM 2.5 mg 3 times/week 4/6–6/2/56
Chlorambucil, 6 mg/day at intervals 1956
Recent treatment, Chlorambucil, 6 mg/day 6/10–7/9/57
Chlorambucil, 6 mg/day 10/16–11/7/57
Hematology, WBC, 95,000; Hg. 14.5 g Plat. C., 150,000
Assay no. 1 10/21/57

L4: A.R. See above
Prior treatment, see above
Recent treatment, Chlorambucil 6 mg/day 3/6–3/27/58
Chlorambucil 6 mg/day 4/3–4/24/58
Hematology, WBC 83,600
Assay no. 2 7/7/58

L5: A.J.R. F 67 Complication, diabetes mellitus, insulin treatment
Treatment, none
Hematology, WBC 35,500; Hg. 13.6 g
Assay no. 1 5/28/58

L6: A.J.R. See above	
Recent treatment, Chlorambucil, 8 mg/day	5/27–6/18/58
Hematology, WBC 5,000	
Assay no. 2	7/7/58
L7: T.S. M 65 Complication, lymphosarcoma of axilla and mediastinum	
Recent treatment, 10 daily treatments of spray X-ray, each 20 r	12/12–12/23/57
Red cell transfusion, 500 ml	12/12/57
Hematology, WBC 487,000, Hg. 10.0 g	
Assay no. 1	12/13/57
L8: T.S. See above	
Recent treatment, 4 X-ray treatments to sarcoma, 40 r each	3/8, 3/10, 3/11 and 3/12/58
Red cell transfusions 500 ml	1/12 and 2/12/58
Testosterone 100 mg 3 times/week	2/12–5/23/58
Hematology, WBC 9,500; Hg. 9.7 g Plat. C., 13,000	
Assay no. 2	3/14/58
L9: T.S. See above	
Recent treatment, red cell transfusions 1500 ml	3/15 and 7/28/58
Testosterone, 50 mg 3 times/week	5/23–7/28/58
Hematology, WBC 22,000	
Assay no. 3	7/28/58
L10: C.L. M 63 Complication, cerebral artery occlusion	
Treatment, none	
Hematology, WBC 19,000; Hg. 14.0 g	
Assay no. 1	10/30/58
L11: A.L. M 50 Complication, diabetes mellitus, Orinase treatment	
Treatment, none	
Hematology, WBC 22,600; Hg. 12.4 g Plat. C., 200,000	
Assay no. 1	10/24/58
L12: T.K. M 73 Complication, bronchopneumonia, antibiotic treatment	
Treatment, Whole blood transfusion 1000 ml	12/13/58
Hematology, WBC 150,000; Hg. 8.3 g	
Assay no. 1	12/19/58
L13: C.V. M 63 Complication, pneumococcal pneumonia, penicillin treatment	
Treatment, none	
Hematology, WBC 60,000; Hct. 44	
Assay no. 1	2/6/59
L14: F.C. M 44	
Prior treatment, TEM intermittent	1951–1959
Recent treatment, TEM 2.5 mg/day	1/15–1/29/59
Hematology, WBC 9,300; Hct. 41.5	
Assay no. 1	2/10/59
L15: M.P. F 79 Complications, staphylococcus pneumonia and arteriosclerotic heart disease, penicillin and digitalis treatment	
Prior treatment, Chlorambucil 12 mg/day for 30 days	1956
X-ray to mediastinum	1/59
Recent, none	
Hematology, WBC 12,500; Hct. 31	
Assay no. 1	3/19/59

L16: L.M. M 67	
Treatment, none	
Hematology, WBC 139,500; Hct. 28	
Assay no. 1	4/13/59
L17: L.M. See above	
Recent treatment, whole blood transfusion 500 ml	4/13/59
Nitrogen mustard 17 mg	4/13 and 4/14/59
Nitrogen mustard 10 mg	4/28/59
Hematology, WBC 57,000; Hct. 35	
Assay no. 2	5/1/59
L18: L.S. F 84	
Recent treatment, whole blood transfusion 2500 ml	5/5–5/8/59
Chlorambucil 10 mg/day	5/6–5/27/59
Cortisone 20 mg/6 hrs	5/6–5/27/59
Hematology, WBC 46,000; Hct. 19	5/8/59
Assay no. 1	
Granulocytic leukemia	
G1: G.B. F 28 Type of leukemia: chronic	
Prior treatment, spray X-ray	1956
Recent treatment, Myleran, 4 mg/day	3/31–4/19/58
Hematology, WBC 236,000; Hg. 13.4 g Plat. C., 360,000	
Assay no. 1	4/2/58
G2: G.B. See above	
Recent treatment, Myleran 2 mg/day	7/30–9/17/58
Hematology, WBC 9,500; Hg. 13.5 g Plat. C., very high	
Assay no. 2	9/17/58
G3: V.M. F 66 Type of leukemia: chronic	
Prior treatment, spray X-ray	1956 and 1957
Recent treatment, none	
Hematology, WBC 19,500; Hg. 10.5 g	
Assay no. 1	9/30/57
G4: J.R. M 45 Type of leukemia: chronic	
Prior treatment, spray X-ray	1956 and 1957
Recent treatment, none	
Hematology, WBC 56,500; Hg. 14.5 g Plat. C., 260,000	
Assay no. 1	11/14/57
G5: E.G. F 14 Type of leukemia: acute	
Complication, leukemic infiltration of lungs	
Prior treatment, multiple whole bloods	1/18–3/17/58
6-Mercaptopurine 25 mg/day	1/18–3/17/58
Aristocort 12 mg/day	1/18–3/18/58
Recent treatment, multiple whole blood transfusions	3/20–5/7/58
6-Mercaptopurine 25 mg/day	3/20–5/7/58
Meticorten 25 mg/day	3/20–5/7/58
Hematology, WBC 7,900; Hg. 10.2 g Plat. C., 20,000	
Assay no. 1	5/7/58
G6: P.R. M 68 Type of leukemia: acute	
Complication, leukemic infiltration of lungs	
Recent treatment, whole blood transfusions 2.0 or 2.5 l per mo	9/25/57–1/8/58
6-Mercaptopurine 50 mg/day	9/25/57–1/8/58
Meticorten 5 mg/day	9/25/57–1/8/58
Testosterone 100 mg/day	1/7/58
X-ray to lungs 50 r	1/7/58
Hematology, WBC 12,000; Hg. 8.2 g Plat. C., 63,000	
Assay no. 1	1/7/58

G7: A.C. F 69 Type of leukemia: subacute	
Precursor condition, polycythemia vera	1953–1955
Complications, diabetes mellitus, Orinase treatment; cholelithiasis	
Prior treatment, X-ray to spleen	1955–1956
Recent treatment, X-ray to spleen	10/16/57
6-Mercaptopurine 25 mg/day	10/17–10/21/57
Hematology, WBC 16,000 Plat. C., low	
Assay no. 1	10/21/57
G8: G.J. F 69 Type of leukemia: subacute	
Precursor condition, polycythemia vera	1955–1957
Complications, diabetes mellitus, insulin treatment; acute pancreatitis; cholelithiasis with operation	10/24/57
Recent treatment, Myleran 12 mg/day	11/7–11/14/57
Red cell transfusions 500 ml	11/6/57
Hematology, WBC 16,000	
Assay no. 1	11/14/57
G9: R.G.H. M 79 Type of leukemia: chronic	
Recent treatment, Myleran 10 mg/day	9/18/58
Hematology, WBC 68,000; Hg. 13.3 g Plat. C., 24,000	
Assay no. 1	9/19/58
G10: R.G.H. See above	
Recent treatment, Myleran 10 mg/day	9/18–10/7/58
Hematology, WBC 6,500; Hg. 13.0 g	
Assay no. 2	11/20/58
G11: R.G.H. See above	
Recent treatment, Myleran 6 mg/day	2/10–3/10/59
Myleran 3 mg/day	3/10–5/1/59
Myleran 2 mg/day	5/1–5/7/59
Hematology, WBC 14,000	
Assay no. 3	5/7/59
G12: C.W. F 43 Type of leukemia: chronic	
Complication, acute streptococcal pharyngitis	12/9/58
Treatment, none	
Hematology, WBC 25,000; Hg. 11.9 g Plat. C., 175,000	
Assay no. 1	12/8/58
G13: J.W. M 60 Type of leukemia: chronic	
Prior treatment, spray X-ray	9/16/58
Recent treatment, none	
Hematology, WBC 46,000; Hg. 10.0 g Plat. C., 50,000	
Assay no. 1	5/8/59
Monocytic leukemia	
M1: P.C. M 79 Type of leukemia: chronic	
Recent treatment, Chlorambucil, 10 mg/day	12/30/57–1/10/58
Hematology, WBC 18,300; Hg. 10.1 g Plat. C., 1,000,000	
Assay no. 1	1/10/58
M2: P.F. M 72 Type of leukemia: acute	
Complication, acute rectal abscess	1/15/59
Treatment, none	
Hematology, WBC 7,550; Hg. 9.7 g	
Assay no. 1	1/13/59

REFERENCES

1. McMenamy, R. H., Lund, C. C., Neville, G. J., and Wallach, D. F. H. Studies of unbound amino acid distributions in plasma, erythrocytes, leukocytes and urine of normal human subjects. J. clin. Invest. 1960, **39**, 0000.
2. Kelley, J. J., and Waisman, H. A. Quantitative plasma amino acid values in leukemic blood. Blood 1957, **12**, 635.
3. Nour-Eldin, F., and Wilkinson, J. F. Amino-acid content of white blood cells in human leukaemias. Brit. J. Haemat. 1955, **1**, 358.
4. Rouser, G. Free or easily extractable amino acids in blood cells and body fluids *in* The Leukemias: Etiology, Pathophysiology and Treatment, J. W. Rebuck, F. H. Bethell, and R. W. Monto, Eds. New York, Academic Press, 1957, p. 361.
5. Iyer, G. Y. N. Paper-chromatographic study of free amino acids in normal and anaemic erythrocytes. Indian J. med. Res. 1957, **45**, 213.
6. Outhouse, E. L. Amino-ethyl phosphoric ester from tumours. Biochem. J. 1936, **30**, 197.
7. White, J. M., Ozawa, G., Ross, G. A. L., and McHenry, E. W. An effect of neoplasms on glutamic acid metabolism in the host. Cancer Res. 1954, **14**, 508.
8. Roberts, E., and Tanaka, T. Free amino acids of the Yoshida ascites tumor. Cancer Res. 1956, **16**, 204.
9. Meister, A. Biochemistry of the Amino Acids. New York, Academic Press, 1957, p. 292.
10. Gardella, J. W., and Lichtler, E. J. The effect of radiation on the nucleic acid, nitrogen, and water content of the Yoshida sarcoma. Cancer Res. 1955, **15**, 529.
11. Klein, G., and Forssberg, A. Studies on the effect of X-rays on the biochemistry and cellular composition of ascites tumors. I. Effect on growth rate, cell volume, nucleic acid and nitrogen content in the Ehrlich ascites tumor. Exp. Cell Res. 1954, **6**, 211.
12. Katz, E. J., and Hasterlik, R. J. Aminoacidurea following total-body irradiation in the human. J. nat. Cancer Inst. 1955, **15**, 1085.
13. Kay, R. E., Early, J. C., and Entenman, C. Increased urinary excretion of taurine and urea by rats after X-irradiation. Radiat. Res. 1957, **6**, 98.
14. Fink, K., Henderson, R. B., and Fink, R. M. Beta-aminoisobutyric acid, a possible factor in pyrimidine metabolism. Proc. Soc. exp. Biol. (N. Y.) 1951, **78**, 135.
15. Awapara, J. Urinary acid excretion in leukemia *in* The Leukemias: Etiology, Pathophysiology, and Treatment, J. W. Rebuck, F. H. Bethell, and R. W. Monto, Eds. New York, Academic Press, 1957, p. 353.

THE ROLE OF PLASMA CO_2 TENSION AND CARBONIC ANHYDRASE ACTIVITY IN THE RENAL REABSORPTION OF BICARBONATE *

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The reabsorption of bicarbonate by the kidneys under normal circumstances appears to be a linear function of the plasma CO_2 tension (1–3). Previous studies from this laboratory (4) demonstrated that when carbonic anhydrase was inhibited by acetazolamide the linear relationship between plasma CO_2 tension and HCO_3^- reabsorption still obtained. It was therefore proposed that the uncatalyzed, as well as the catalyzed, hydration of CO_2 was an important source of the H^+ involved in the reabsorption of HCO_3^-.

The present investigations were undertaken in an attempt to characterize more precisely the catalyzed and uncatalyzed reactions. By varying plasma CO_2 tension, carbonic anhydrase activity, and filtered HCO_3^-, three aspects of HCO_3^- reabsorption were examined: *1*) the maximal reabsorptive capacity, or the HCO_3^- Tm, with and without carbonic anhydrase activity; *2*) the relation of HCO_3^- excretion to HCO_3^- Tm with and without carbonic anhydrase activity; *3*) the capacity of high plasma CO_2 tensions to effect complete HCO_3^- reabsorption in the absence of carbonic anhydrase.

On the basis of these studies it was concluded that HCO_3^- reabsorption is mediated by two distinct processes. One process has a HCO_3^- Tm which is dependent on plasma pCO_2 and independent of carbonic anhydrase activity. The second process is dependent on carbonic anhydrase, independent of plasma pCO_2, and necessary for the establishment of sharp pH gradients between blood and urine.

METHODS

Experiments were performed on female dogs anesthetized with either Nembutal, sodium pentothal, or Fluothane. An endotracheal tube, fitted with an inflatable cuff, was inserted into the trachea and connected to a Bird assisted-respiratory anesthesia unit. Respiratory movements were inhibted by either d-tubocurare, succinylcholine, or gallium triethiodide (Flaxedil) to facilitate control of rate and depth of ventilation with the respirator. The concentration of CO_2 in inspired air varied by controlling the flow rate of 100 per cent CO_2 and 100 per cent O_2 into the respirator. In some experiments alveolar pCO_2 was monitored with a Liston-Becker infrared CO_2 analyzer.

To determine the time required for HCO_3^- reabsorption to stabilize after an abrupt change in plasma pCO_2, 2 dogs were studied for 7 periods each. Plasma pCO_2 was abruptly elevated to approximately 150 mm Hg and then maintained at this level. Bicarbonate reabsorption reached a stable value within 10 minutes and remained constant for as long as 2 hours. A 15 minute equilibration period was chosen in order not to prolong unnecessarily the length of the experiment.

In the first group of experiments on 15 normal dogs, the effect of pCO_2 on the maximal HCO_3^- reabsorptive capacity (HCO_3^- Tm) was studied. Plasma HCO_3^- concentration was elevated by the injection of 12 g $NaHCO_3$ and maintained at a high level by the constant infusion of isotonic $NaHCO_3$ at the rate of approximately 10 ml per minute throughout the experiment. Plasma pCO_2 was varied in 10 dogs from extreme respiratory alkalosis (pCO_2, 6 mm Hg) to extreme respiratory acidosis (pCO_2, 400 mm Hg) by either hyperventilation or by changing the concentration of CO_2 in the inspired air. In 6 of these dogs the study was repeated after the administration of acetazolamide, 50 mg per kg body weight. In 5 additional dogs the studies were performed only after the administration of acetazolamide. The kidneys from 4 of the dogs given acetazolamide were removed at the termination of the experiment, weighed, and assayed for carbonic anhydrase activity.

In the second group of experiments the relationship of HCO_3^- excretion to HCO_3^- reabsorption was studied as plasma HCO_3^- concentration was progressively

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elevated from low values to levels at which a HCO_3^- Tm was demonstrated. Plasma pCO_2 was maintained constant at normal or elevated levels with and without acetazolamide administration. In 3 dogs pCO_2 was maintained at normal levels (33 to 40 mm Hg) by breathing room air. In 4 dogs pCO_2 was maintained at 85 to 100 mm Hg by breathing 9 per cent CO_2 and 91 per cent O_2. Eight additional dogs were studied at the normal (4 dogs) and elevated (4 dogs) tensions of CO_2 after the administration of acetazolamide (prime dose 50 mg per kg body weight; sustaining infusion, 1 mg per minute). After plasma pCO_2 was stabilized for about 1 hour, plasma HCO_3^- concentration was slowly elevated in stepwise fashion from depressed values by injections of 6 per cent $NaHCO_3$. After each injection an equilibration period of 15 to 20 minutes was permitted before beginning a collection period.

In the third group of experiments on 4 dogs a mild metabolic acidosis was induced by administration of 10 to 15 g NH_4Cl on the day preceding the experiment. Acetazolamide (25 to 50 mg per kg body weight) was administered to inhibit carbonic anhydrase activity. The plasma pCO_2 was then progressively elevated in an attempt to obliterate HCO_3^- excretion.

Urines were collected in oiled syringes through an indwelling catheter at the mid-point of the collection period for measurement of pH and CO_2 content. At the end of the collection period the bladder was emptied by manual compression and washed with 20 ml distilled water. Heparinized blood samples were drawn anaerobically from the femoral artery. Methods used were those previously described (4).

To determine the extent to which renal carbonic anhydrase was inhibited by the administration of 50 mg per kg of acetazolamide, the kidneys were removed at the end of the experiment and perfused with 300 to 500 ml of ice-cold isotonic saline to remove all red cells. In addition, the saline perfusion served to wash acetazolamide out of the intravascular and interstitial spaces, and also out of the tubular lumen, thus minimizing enzyme inhibition by acetazolamide not located within renal tubular cells. The kidneys were then homogenized in ice-cold distilled water, diluted 1 : 50, 1 : 100 and 1 : 1,000 and assayed by the method of Davis (5). With this method the addition of 1 ml of 1 : 1,000 solution of normal kidney to 100 ml of reaction solution produced a fivefold increase in the rate constant for the hydration of CO_2, whereas the addition of 200 times this quantity of kidney (10 ml of 1 : 50 solution of kidney) from a dog given 50 mg acetazolamide per kg body weight had no measurable effect on the rate constant.

RESULTS

I. Effect of plasma CO_2 tension on the HCO_3^- Tm

To study the effects of plasma CO_2 tension on the HCO_3^- Tm, plasma HCO_3^- concentration was raised to a level such that filtered HCO_3^- always greatly exceeded the HCO_3^- Tm. Schwartz, Falbriard and Lemieux (6) have presented data suggesting that the HCO_3^- Tm is approached gradually during acute respiratory acidosis. Data presented below (Figure 4), however, show that at CO_2 tensions ranging between 85 and 100 mm Hg a Tm of 3.7 mEq per L was obtained when plasma HCO_3^- concentration (corrected for Donnan factor) reached 41 mEq per L [i.e., filtered HCO_3^- per unit glomerular filtrate (GF) exceeded the Tm by approximately 15 per cent]. Similarly, at still higher CO_2 tensions (150 to 200 mm Hg) a Tm was obtained when filtered HCO_3^- per unit GF exceeded the Tm by only 10 to 12 per cent (7). With the exception of the experiment on Dog 4 (which was designed primarily to study HCO_3^- reabsorption during respiratory alkalosis) the concentration of HCO_3^- in GF always exceeded the HCO_3^- Tm by at least 25 per cent. In animals given acetazolamide, however, the HCO_3^- Tm was reached only when the filtered HCO_3^- was approximately twice the Tm at normal CO_2 tensions and about 1.5 to 1.8 times the Tm at elevated CO_2 tensions (Figure 5). Therefore, to insure valid Tm measurements in the presence of carbonic anhydrase inhibition, the concentration of HCO_3^- in GF was always maintained at a level 2.5 to 5.0 times greater than the HCO_3^- Tm.

A. Intact carbonic anhydrase enzyme system. In 10 dogs the plasma pCO_2 was varied from 6 to 400 mm Hg. The first two protocols of Table I summarize representative experiments. Figure 1 depicts the data from all the studies. The lowest plasma pCO_2 was 6 mm Hg, a level at which HCO_3^- reabsorption was still significant (1.4 mEq per 100 ml GF). Owing to the invariable appearance of pulmonary edema and hemolysis it was impossible to study HCO_3^- reabsorption below 6 mm Hg. For this reason the intercept value at zero CO_2 tension could not be identified. Although the over-all shape of the curve suggests that it might project through the origin, it could equally well intercept the vertical axis at some point above the origin.

Unlike previous studies (1–3), it is apparent from Figure 1 that increasing plasma pCO_2 accelerates HCO_3^- reabsorption in curvilinear fashion. Although the curve tends to become flat at higher CO_2 tensions, a point was never reached

TABLE I

The effect of CO_2 tension and carbonic anhydrase activity on the maximal bicarbonate reabsorptive capacity *

Time	Treatment	Plasma			Urine			Bicarbonate		
		HCO_3^-	pH	pCO_2	C_{in}	Flow	pH	Filt.	Excr.	Reab.
min		*mEq/L*		*mm Hg*	*ml/min*	*ml/min*		*μEq/min*	*μEq/min*	*mEq/100 ml GF*
Dog 4; wt. 30 kg										
0	Anesthesia, sodium pentothal and succinylcholine chloride; infuse 0.15 M $NaHCO_3$ at 10 ml/min									
60–70	Hyperventilation	24.3	7.83	14	60.8	1.66	8.26	1,475	354	1.84
70–80	Hyperventilation	22.5	7.89	11	70.6	1.18	8.49	1,585	184	1.98
80–90	Hyperventilation	21.4	7.93	10	80.5	1.30	8.49	1,725	153	1.95
105–115	Breathing room air	33.7	7.56	42	100	2.70	7.90	3,370	363	3.01
130–140	Breathing 9% CO_2	41.5	7.31	81	87.5	1.93	7.75	3,630	251	3.87
155–165	Breathing 16% CO_2	45.3	7.14	131	83.7	1.56	7.63	3,790	261	4.22
180–195	Breathing 23% CO_2	50.0	7.13	148	81.5	2.20	7.39	4,065	375	4.53
Dog 11; wt. 12 kg										
0	Anesthesia, sodium pentothal and succinylcholine chloride; prime 12 g Na HCO_3; infuse 0.15 M $NaHCO_3$ at 10 ml/min									
30–40	Hyperventilation	40.7	7.73	30	28.9	7.34	8.03	1,178	543	2.20
55–65	Breathing room air	46.1	7.58	48	31.0	8.34	7.75	1,430	547	2.85
80–90	Breathing 5% CO_2	54.2	7.54	62	29.5	8.07	7.72	1,595	695	3.05
105–115	Breathing 9% CO_2	61.0	7.45	86	30.0	7.47	7.65	1,830	775	3.53
130–140	Breathing 17% CO_2	66.8	7.29	136	30.0	6.48	7.55	2,005	715	4.29
155–165	Breathing 23% CO_2	68.4	7.20	172	29.7	6.22	7.36	2,030	670	4.58
180–190	Breathing 29% CO_2	70.8	7.12	214	28.7	6.69	7.29	2,030	700	4.63
206	Acetazolamide 600 mg i.v.; infuse 0.15 M $NaHCO_3$ at 10 ml/min and acetazolamide at 2 mg/min									
235–245	Breathing room air	67.7	7.73	50	21.5	11.1	7.86	1,458	1,200	1.20
260–270	Breathing 5% CO_2	71.0	7.52	85	21.3	10.2	7.63	1,512	1,088	1.99
285–295	Breathing 9% CO_2	74.9	7.48	99	20.9	9.68	7.56	1,565	1,075	2.34
310–320	Breathing 17% CO_2	76.2	7.32	145	20.2	9.21	7.51	1,540	1,005	2.65
335–345	Breathing 23% CO_2	78.9	7.28	165	18.1	8.44	7.44	1,425	930	2.75
360–370	Breathing 29% CO_2	80.3	7.16	221	19.6	8.34	7.27	1,510	965	3.09
385–393	Breathing 37% CO_2	80.1	7.06	279	18.7	8.52	7.18	1,500	935	3.22

* In Tables I–IV plasma bicarbonate concentrations have been corrected for a Donnan factor of 1.05.

at which additional increments in pCO_2 did not elicit further increases in HCO_3^- reabsorption. The straight line relationship previously reported is doubtless the result of the limited range over which plasma pCO_2 was varied.

B. Inhibited carbonic anhydrase enzyme system. In 11 dogs the HCO_3^- Tm was examined while the plasma pCO_2 was varied from 30 to 350 mm Hg after injection of large doses of acetazolamide. A sample protocol is presented in Table I; the data from all experiments are plotted in Figure 2.

Inhibition of red cell carbonic anhydrase prevented the lowering of the plasma pCO_2 below 30 mm Hg. At any given concentration of CO_2 in the inspired air, arterial pCO_2 averaged 20 to 40 mm Hg higher during acetazolamide administration than under normal circumstances.

The administration of acetazolamide tended to give somewhat scattered results when different animals were compared (Figure 2). To circumvent variations arising from different responses of dogs to acetazolamide, six dogs were studied over the complete range of plasma pCO_2 while carbonic anhydrase was intact and then restudied after administration of acetazolamide. The data from single animals generated remarkably smooth curves (Figure 3). The curve obtained after carbonic anhydrase inhibition is also curvilinear, in contrast to the straight line obtained in the previous study (4) where the plasma pCO_2 was varied over a much smaller range.

At the conclusion of the experiments, analysis of the kidneys revealed no demonstrable evidence of carbonic anhydrase activity. The absence of carbonic anhydrase activity in the kidneys of dogs

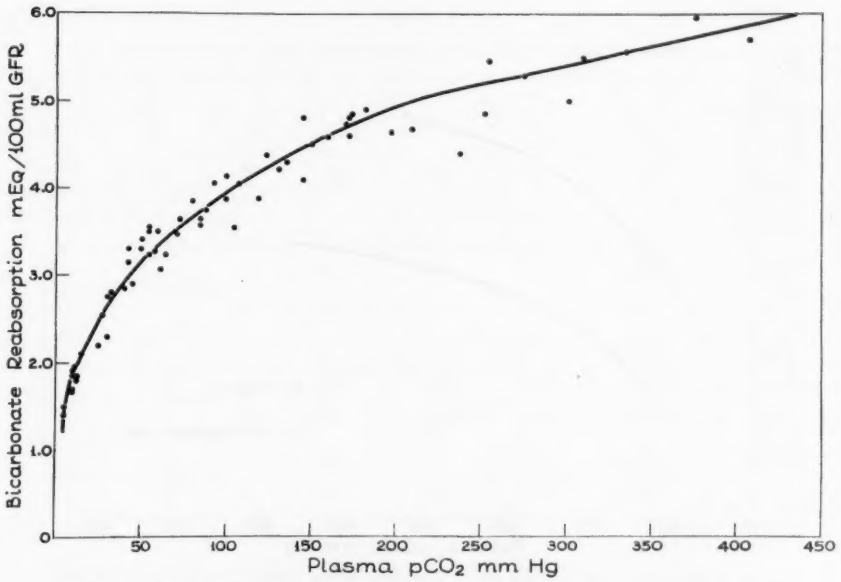

FIG. 1. RELATION BETWEEN PLASMA pCO_2 AND HCO_3^- REABSORPTION IN DOGS.

given acetazolamide cannot be cogently attributed to artifacts of the assay method. The extensive perfusion of the kidney before homogenization minimized contamination of intracellular enzyme with extracellular inhibitor. In addition, the assay method tends to err in the direction of exaggerating, not underestimating, enzyme activity, owing to dissociation of the enzyme-inhibitor complex in diluted kidney homogenates. This direct evidence of complete inhibition is consonant with the results of the recent kinetic analysis of carbonic anhydrase inhibition developed by Maren, Tarcell and Malik (8). These investigators have shown that the ratio of free enzyme [E] to inhibited enzyme [EI] is given by the expression:

$$\frac{[E]}{[EI]} = \frac{K_I}{[I]}$$

where K_I for acetazolamide is 8×10^{-9}M and [I] is the tissue concentration of free acetazolamide. The administration of 50 mg per kg acetazolamide produces a plasma concentration of approximately 5×10^{-4} M; and Maren, Wadsworth, Yale and Alonso have shown the concentration of free inhibitor in cellular water to be the same as that in plasma (9). Therefore, $\frac{[E]}{[EI]}$ is 1/50,000; consequently the per cent inhibition is 99.998. Finally, the demonstration that doses of acetazolamide above 20 mg per kg elicit no further response strongly supports (although it does not by itself conclusively establish) the contention that the physiologic effects of carbonic anhydrase are completely blocked (9, 10).

II. Bicarbonate excretion as HCO_3^- Tm is approached

A. Normal plasma pCO_2 (33 to 40 mm Hg). In dogs with a normal plasma pCO_2, a HCO_3^- Tm

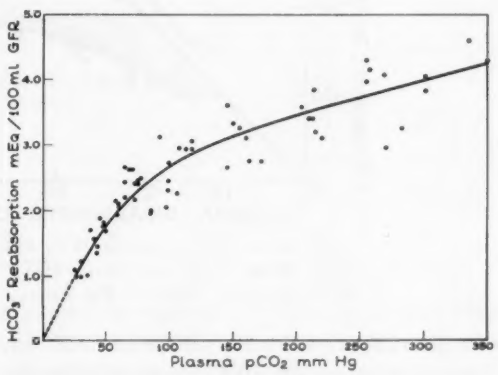

FIG. 2. EFFECT OF ACETAZOLAMIDE ON THE RELATION BETWEEN HCO_3^- REABSORPTION AND PLASMA pCO_2.

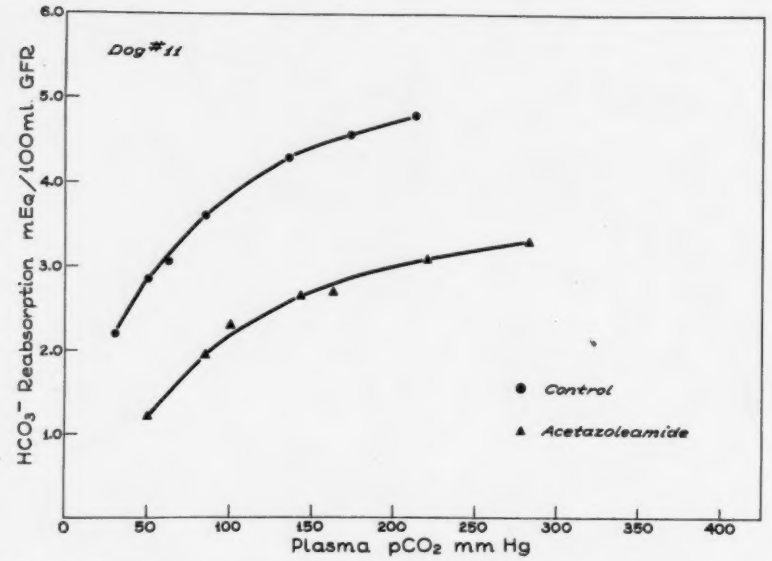

FIG. 3. COMPARISON OF THE RELATION BETWEEN HCO_3^- REABSORPTION AND PLASMA pCO_2 WITH AND WITHOUT ACETAZOLAMIDE.

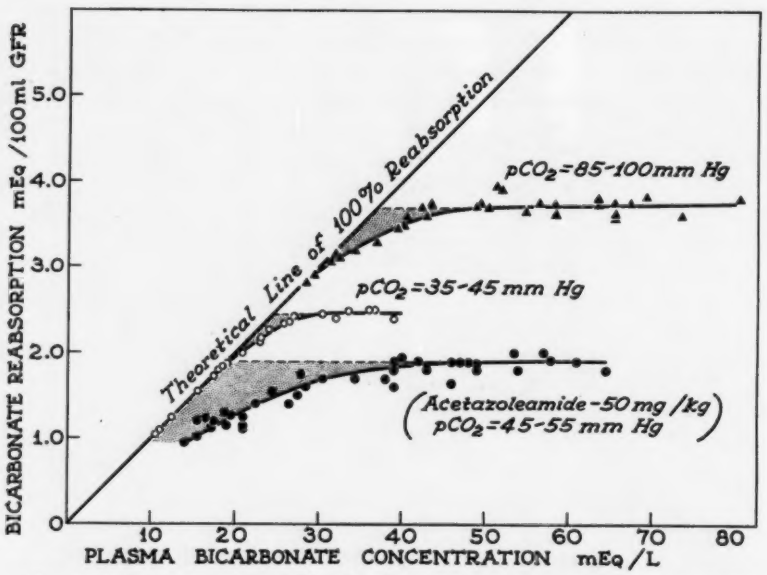

FIG. 4. EFFECT OF PLASMA pCO_2 AND ACETAZOLAMIDE ON THE RELATION BETWEEN HCO_3^- REABSORPTION AND PLASMA HCO_3^- CONCENTRATION. The stippled area indicates HCO_3^- excretion before the Tm was reached, and is termed a HCO_3^- leak (see text).

of approximately 2.6 mEq per 100 ml GF was obtained when plasma HCO_3^- concentration was progressively increased (Table II, Dog 72; Figure 4, middle curve). The stippled area on the middle curve of Figure 4 indicates that a small amount of HCO_3^- escaped reabsorption before the Tm was reached.

B. Elevated plasma pCO_2 (85 to 100 mm Hg).

TABLE II

The relationship of bicarbonate excretion to plasma bicarbonate concentration

Time	Plasma			C_{in}	Urine		Bicarbonate		
	HCO_3^-	pH	pCO_2		Flow	pH	Filt.	Excr.	Reab.
min	*mEq/L*		*mm Hg*	*ml/min*	*ml/min*		*μEq/min*	*μEq/min*	*mEq/100 ml GF*
Normal pCO_2: Dog 72; wt. 28 kg									
0	Anesthesia, sodium pentothal; breathing room air; infuse isotonic saline								
60–80	18.9	7.34	34	84	4.02	6.97	1,585	31	1.85
85	Inject 1.5 g $NaHCO_3$								
100–110	22.8	7.44	33	75	3.35	7.27	1,712	70	2.19
115	Inject 1.5 g $NaHCO_3$								
130–140	23.5	7.45	35	62	3.11	7.37	1,520	106	2.28
145	Inject 1.5 g $NaHCO_3$; infuse $NaHCO_3$, 250 μEq/min								
160–170	26.3	7.45	37	78	4.81	7.62	2,060	240	2.34
175	Inject 1.5 g $NaHCO_3$								
190–200	27.1	7.53	33	65	3.59	7.56	1,765	197	2.41
205	Inject 3.0 g $NaHCO_3$								
220–230	33.5	7.55	38	64	7.33	7.71	2,145	585	2.46
235	Inject 3.0 g $NaHCO_3$								
250–260	36.8	7.56	40	60	7.64	7.75	2,205	704	2.50
Elevated pCO_2: Dog 53; wt. 14.5 kg									
0	Anesthesia, continuous 1.5% Fluothane; breathing 8% CO_2 and 92% O_2								
30	Prime 12 g $NaHCO_3$; infuse 500 μEq $NaHCO_3$/min i.v.								
60–75	40.1	7.28	85	67	2.44	7.65	2,638	350	3.49
75–90	39.7	7.26	87	59	2.47	7.61	2,319	303	3.45
91	Prime 12 g $NaHCO_3$; infuse 750 μEq $NaHCO_3$/min i.v.								
120–135	49.5	7.34	90	58	4.36	7.67	2,857	680	3.77
135–150	49.2	7.35	87	56	4.25	7.68	2,757	638	3.70
151	Prime 12 g $NaHCO_3$; infuse 1,000 μEq $NaHCO_3$/min i.v.								
180–195	58.3	7.43	86	60	7.17	7.74	3,484	1,232	3.76
195–210	58.4	7.44	85	55	6.86	7.75	3,196	1,213	3.62
211	Prime 12 g $NaHCO_3$; infuse 1,250 μEq $NaHCO_3$/min i.v.								
240–255	65.3	7.46	90	54	8.75	7.74	3,529	1,530	3.70
255–270	64.9	7.46	90	56	9.62	7.76	3,635	1,643	3.56
Elevated pCO_2: Dog 70; wt. 14 kg									
0	Anesthesia, sodium pentothal and d-tubocurare; breathing 7% CO_2 and 93% O_2								
30	Infuse isotonic saline								
90–115	23.3	7.04	85	55.8	0.67	5.30	1,305		2.33
120	Inject 3 g $NaHCO_3$								
130–140	29.7	7.13	88	60.5	2.63	6.91	1,805	55	2.89
145	Infuse 250 μEq $NaHCO_3$/min								
155–165	28.5	7.15	82	63.3	3.56	6.83	1,810	56	2.78
170	Inject 1.5 g $NaHCO_3$								
180–195	32.5	7.21	80	59.0	3.46	7.12	1,905	107	3.08
200	Inject 1.5 g $NaHCO_3$								
210–220	34.5	7.23	81	58.5	3.06	7.38	2,015	155	3.18
225	Inject 1.5 g $NaHCO_3$; increase $NaHCO_3$ infusion to 500 μEq/min								
235–245	36.6	7.25	82	59.2	2.77	7.52	2,170	223	3.29
250	Inject 1.5 g $NaHCO_3$								
265–275	40.8	7.29	83	58.2	4.00	7.63	2,380	285	3.60
280	Inject 1.5 g $NaHCO_3$								
290–300	42.2	7.29	86	56.4	5.17	7.69	2,370	302	3.69
305	Inject 6.0 g $NaHCO_3$								
315–325	50.4	7.38	85	55.0	7.80	7.58	2,775	740	3.70

During respiratory acidosis HCO_3^- reabsorption was increased to a maximal value of approximately 3.8 mEq per 100 ml GF (Table II, Dog 53; Figure 4, upper curve). That this value in fact represents a true HCO_3^- Tm is evidenced by the constancy of reabsorption when the filtered load of HCO_3^- was increased from 4.2 to 8.0 mEq per 100 ml GF. As previously shown by others (6), HCO_3^- excretion began before the HCO_3^- Tm was reached (Table II, Dog 70; Figure 4). The pat-

tern of the HCO_3^- leak, although greater in amount, was similar to that seen at normal CO_2 tensions.

C. Normal plasma pCO_2 plus acetazolamide. Inhibition of carbonic anhydrase by the administration of acetazolamide (50 mg per kg body weight) in amounts sufficient to produce maximal physiologic effects depressed the HCO_3^- Tm to 1.9 mEq per 100 ml GF (Table III, Dog 63; Figure 4, lower curve). Large quantities of HCO_3^- were excreted before the Tm was reached. The magnitude of the HCO_3^- leak is significantly greater than that seen at either normal or elevated CO_2 tensions. These results are similar to those obtained by Schwartz, Falbriard, and Relman (10) in that the relation between plasma HCO_3^- concentration and HCO_3^- reabsorption is curvilinear, but differ in that a distinct HCO_3^- Tm was obtained.

D. Elevated plasma pCO_2 plus acetazolamide. It has already been demonstrated (4) that HCO_3^- reabsorption increases linearly as pCO_2 is raised. If, therefore, carbonic anhydrase inhibition lowers the Tm and augments the HCO_3^- leak because of inadequate H^+ production, it should be possible to overcome the deficient production by raising plasma CO_2 tension. Increasing pCO_2 from nor-

TABLE III

The effect of acetazolamide on bicarbonate reabsorption at various concentrations of plasma bicarbonate

Time	Plasma			Cin	Urine		Bicarbonate		
	HCO_3^-	pH	pCO_2		Flow	pH	Filt.	Excr.	Reab.
min	*mEq/L*		*mm Hg*	*ml/min*	*ml/min*		*μEq/min*	*μEq/min*	*mEq/100 ml GF*
Normal pCO_2; Dog 63; wt. 10 kg									
0	Anesthesia, sodium pentothal; breathing room air; injection, 250 mg acetazolamide; continuous infusion 0.15 M NaCl at 5 ml/min + acetazolamide 1.0 mg/min								
30–45	22.9	7.31	44	23.9	0.91	7.94	548	214	1.40
46	Inject 3 g $NaHCO_3$; infuse 250 μEq/min $NaHCO_3$ + 1.0 mg/min acetazolamide								
55–70	28.0	7.39	45	30.1	4.4	7.60	845	323	1.70
71	Inject 3 g $NaHCO_3$								
80–95	34.1	7.48	45	29.7	7.0	7.57	1,012	496	1.74
96	Inject 3 g $NaHCO_3$								
105–120	39.4	7.53	46	31.6	8.9	7.65	1,245	639	1.91
121	Inject 3 g $NaHCO_3$; increase $NaHCO_3$ infusion to 500 μEq/min; continue acetazolamide at 1.0 mg/min								
130–145	42.9	7.58	44	26.1	7.3	7.72	112	615	1.93
146	Inject 6 g $NaHCO_3$								
155–170	54.0	7.61	52	22.5	6.6	7.78	1,215	800	1.85
171	Inject 6 g $NaHCO_3$								
180–195	64.5	7.65	56	23.5	8.1	7.78	1,515	1,069	1.88
Elevated pCO_2: Dog 77; wt. 20 kg									
0	Anesthesia, sodium pentothal and Flaxedil; breathing 9% CO_2 and 91% O_2								
30	Inject 1 g acetazolamide; infuse isotonic saline and acetazolamide at 1 mg/min								
60–80	23.6	6.93	111	52.4	1.08	7.58	1,237	208	1.96
85	Inject 3 g $NaHCO_3$								
95–105	27.7	6.98	115	53.2	2.35	7.48	1,472	373	2.07
110	Inject 3.6 g $NaHCO_3$								
120–130	31.7	7.03	118	57.7	3.37	7.47	1,832	465	2.37
135	Inject 3.6 g $NaHCO_3$; infuse $NaHCO_3$ 250 μEq/min and acetazolamide 1 mg/min								
145–155	35.0	7.05	125	54.3	3.71	7.49	1,902	500	2.58
160	Inject 5.4 g $NaHCO_3$								
170–180	41.5	7.12	125	50.2	5.37	7.51	2,083	826	2.50
185	Inject 5.4 g $NaHCO_3$								
195–205	46.2	7.15	130	54.1	6.38	7.52	2,498	1,026	2.72
210	Inject 5.4 g $NaHCO_3$								
220–230	48.3	7.18	117	58.8	7.09	7.51	2,838	1,220	2.75
235	Inject 7.2 g $NaHCO_3$								
245–255	55.3	7.21	134	57.7	8.50	7.52	3,186	1,571	2.80
260	Inject 7.2 g $NaHCO_3$								
270–280	59.0	7.27	127	53.1	8.77	7.55	3,130	1,680	2.75
285	Inject 9.0 g $NaHCO_3$								
295–305	67.5	7.32	126	43.8	8.92	7.59	2,960	1,744	2.78

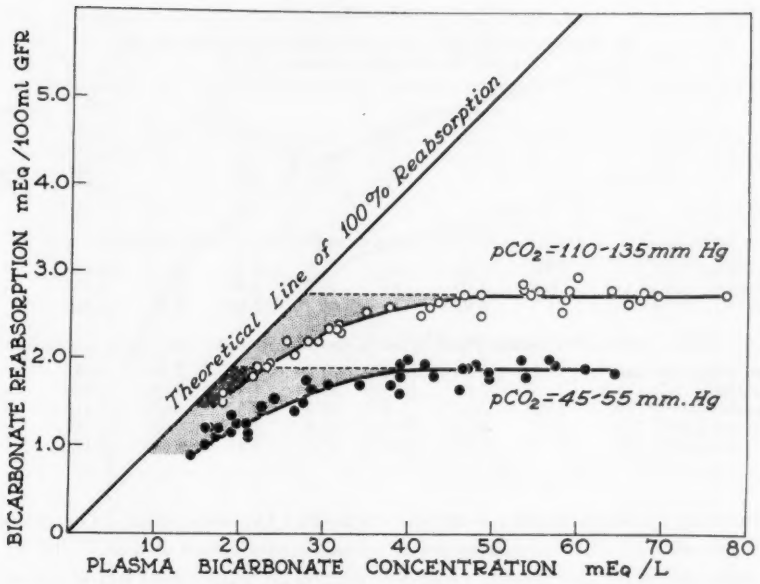

FIG. 5. FAILURE OF RESPIRATORY ACIDOSIS TO ELIMINATE THE HCO_3^- LEAK INDUCED BY ACETAZOLAMIDE.

mal values to 110 to 130 mm Hg increased the HCO_3^- Tm from 1.9 to approximately 2.8 mEq per 100 ml GF but did not diminish the magnitude of the HCO_3^- leak (Table III, Dog 77; Figure 5, upper curve). It would appear, therefore, that in the absence of carbonic anhydrase, raising the plasma pCO_2 can restore HCO_3^- Tm to a normal value, but cannot diminish the HCO_3^- leak.

III. Effect of raising plasma pCO_2 on an acetazolamide-induced HCO_3^- diuresis during metabolic acidosis

The demonstration in the first group of experiments that the reabsorption of HCO_3^-, despite carbonic anhydrase inhibition, could be increased by raising pCO_2 formed the basis for the third group of experiments. In these studies a mild metabolic acidosis was induced, carbonic anhydrase was inhibited with acetazolamide and a HCO_3^- diuresis ensued. Attempts were then made to obliterate the HCO_3^- diuresis by increasing plasma pCO_2. A representative protocol is presented in Table IV.

During the control period when plasma pCO_2 was normal, HCO_3^- reabsorption was virtually complete. Raising plasma pCO_2 from 36 to 113 mm Hg increased both filtered and reabsorbed HCO_3^- without altering excretion. Simultaneous blood and urine pH's both fell, with a proportionately greater drop in urine pH. Respiratory acidosis, however, failed to elicit the maximal urinary acidity that has been observed in the dog during severe metabolic acidosis (11).

Following the administration of acetazolamide, HCO_3^- excretion rose to 249 μEq per minute. Raising plasma CO_2 tension from 36 to 184 mm Hg in stepwise fashion increased HCO_3^- reabsorption, but had only a modest effect on HCO_3^- excretion. The fall in HCO_3^- excretion to 76 μEq per minute in the last period was in part the result of a fall in glomerular filtration rate (GFR) from 61 to 42 ml per minute. Blood and urine pH were both reduced as pCO_2 was increased, but in the face of carbonic anhydrase inhibition, urine pH never fell below that of blood.

DISCUSSION

The reabsorption of $NaHCO_3$ is regarded as the consequence of the secretion of cellular H^+ in exchange for tubular Na^+ (11, 12). Two of the principal determinants of H^+ secretion thus far identified are the CO_2 tension of plasma (1–3)

TABLE IV

The effect of elevated pCO_2 on acetazolamide-induced bicarbonate diuresis during mild metabolic acidosis

Time	Treatment	Plasma			C_{In}	Urine		Bicarbonate		
		HCO_3^-	pH	pCO_2		Flow	pH	Filt.	Excr.	Reab.
min		*mEq/L*		*mm Hg*	*ml/min*	*ml/min*		*μEq/min*	*μEq/min*	*mEq/100 ml GF*
Dog 28; wt. 13.7 kg										
0	Anesthesia, sodium pentothal; infuse 5% dextrose in distilled H_2O at 5 ml/min									
60–70	Breathing room air	16.6	7.26	36	67.9	5.42	6.56	1,125	10	1.65
85–95	Breathing 9% CO_2	20.5	7.09	67	72.8	5.85	6.48	1,490	16	2.02
110–120	Breathing 23% CO_2	25.3	6.95	113	60.7	2.98	6.14	1,535	5	2.53
121	Acetazolamide 350 mg i.v.; infuse 5% dextrose in distilled H_2O at 5 ml/min + acetazolamide 1 mg/min									
140–150	Breathing room air	17.7	7.29	36	61.2	7.23	7.38	1,085	249	1.37
165–175	Breathing 16% CO_2	23.5	7.00	93	57.8	4.22	7.10	1,360	143	2.11
190–200	Breathing 23% CO_2	25.3	6.93	119	57.7	3.54	7.15	1,460	177	2.23
215–225	Breathing 29% CO_2	27.3	6.84	153	56.8	3.65	6.93	1,550	146	2.48
240–250	Breathing 38% CO_2	27.8	6.78	185	42.3	2.43	6.79	1,175	74	2.60

and the activity of the carbonic anhydrase enzyme system (11). Plasma pCO_2 is thought to influence HCO_3^- reabsorption by altering the production of H^+ ions, predominantly via the catalyzed hydration of CO_2. In the present studies, however, inhibition of carbonic anhydrase did not prevent the augmentation of HCO_3^- reabsorption when plasma pCO_2 was increased. In fact, the regulatory effect of pCO_2 on the capacity to reabsorb HCO_3^- appeared to be entirely independent of carbonic anhydrase activity.

A comparison of the relationship between maximal HCO_3^- reabsorptive capacity and plasma CO_2 tension in the presence and absence of carbonic anhydrase activity (Figure 6) clearly discloses that the difference between the upper (carbonic anhydrase intact) and lower (carbonic anhydrase inhibited) curves is constant at all CO_2 tensions studied. This means that the contribution of the carbonic anhydrase enzyme system to HCO_3^- reabsorption is independent of plasma pCO_2 and amounts to 1.4 mEq per 100 ml GF, as is shown in the inset of Figure 6. It also follows from an analysis of this figure that since an increase in pCO_2 accelerates HCO_3^- reabsorption to the same extent in the presence or absence of carbonic anhydrase activity, its action is mediated entirely by the uncatalyzed hydration of CO_2.

To determine whether the uncatalyzed hydration of CO_2 could, in fact, account for all HCO_3^- reabsorption observed in the absence of carbonic anhydrase activity, calculations similar to those of Davies (13) were used. The calculated rates of H_2CO_3 production at a pCO_2 of 40 mm Hg [assuming an intracellular pH of 7.0 to 7.2, an intracellular HCO_3^- concentration of 2.6 to 5.0 mEq per L, and a rate constant, K_{CO_2}, for the reaction $CO_2 + H_2O \rightleftarrows H_2CO_3$, of 0.11 second^{-1} at 38° C (14)] varied from 0.06 to 0.13 μmoles per second per ml intracellular water. The observed rates of HCO_3^- reabsorption at CO_2 tensions of 40 mm Hg and plasma HCO_3^- concentrations of approximately 50 mEq per L were 0.33 μEq per second per ml of estimated intracellular water.[1] Thus the observed rates of HCO_3^- reabsorption were three to five times greater than the calculated rates of H_2CO_3 production.

The discrepancy between the calculated rate of H_2CO_3 production and the observed rate of HCO_3^- reabsorption suggests that some source other than the hydration of CO_2 was contributing H^+ to the transport process. In Figure 2, extrapolation of the curve to zero CO_2 tension intercepts the ordinate at approximately the origin. While such an extrapolation is admittedly a crude estimate of HCO_3^- reabsorption at zero CO_2 tension (i.e., the intercept could be either slightly above or slightly below the origin), it does indicate that

[1] Intracellular water was estimated by removing the kidneys from four dogs previously given acetazolamide. The kidneys were then weighed and dried to determine total kidney water. Intracellular water was assumed to be 50 per cent of the total kidney water because of the relatively higher extracellular fluid volume of the kidney.

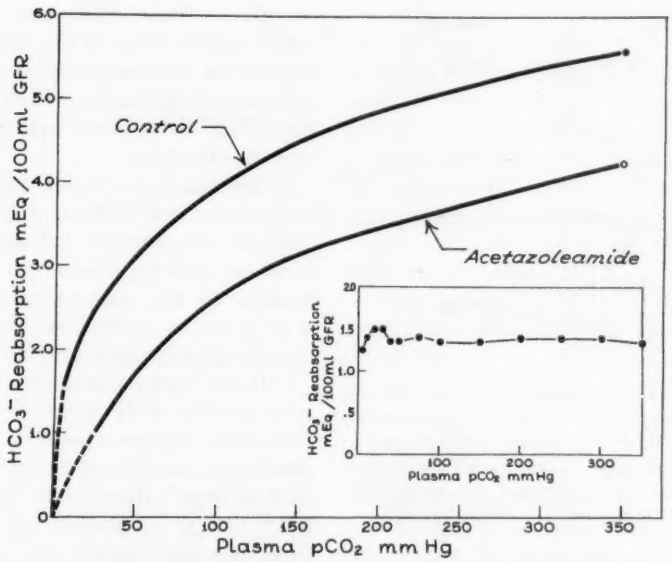

FIG. 6. COMPARISON OF THE RELATION BETWEEN HCO_3^- REABSORPTION AND PLASMA pCO_2 WITH AND WITHOUT ACETAZOLAMIDE. The upper curve (carbonic anhydrase intact) was taken from Figure 1, while the lower curve (carbonic anhydrase inhibited) was taken from Figure 2. Complete inhibition of carbonic anhydrase reduces the HCO_3^- Tm by a constant amount at all CO_2 tensions so that the lower curve parallels the upper curve. The intercept point, while not precisely defined for the upper curve, approximates the origin for the lower curve. . The contribution of the carbonic anhydrase enzyme system at all levels of plasma pCO_2 is plotted in the inset as the difference between the upper and lower curves.

most, if not all, HCO_3^- reabsorption is in some way dependent upon CO_2 when carbonic anhydrase is maximally inhibited. Therefore, if other metabolic processes contribute H^+ to HCO_3^- reabsorption, their contribution is relatively minor and certainly not nearly of sufficient magnitude to account for the three- to fivefold calculated deficit in H^+ production.

Actually it is not necessary to postulate a special source of H^+ to compensate for the deficit in H_2CO_3 production from CO_2. The reabsorption of HCO_3^- secondary to H^+ secretion involves the formation of H_2CO_3 in the tubular lumen, some of which will return to the cell, contributing directly to the supply of intracellular H_2CO_3. Figure 7 depicts the two mechanisms whereby H^+ secretion, in the process of mediating HCO_3^- reabsorption, leads to the return of non-ionized H_2CO_3 to the tubular cell. First, as in the case of other undissociated acids, H_2CO_3 will back-diffuse into the cell by a process of non-ionic diffusion through the lipoid luminal membrane. Second, the reabsorption of large amounts of filtrate will sweep H_2CO_3 back into the cell through aqueous-filled pores as a result of solvent drag. This recycling of H_2CO_3 back into the cell completes the process of HCO_3^- reabsorption initiated by the secretion of H^+ and at the same time markedly reduces the need for high rates of H_2CO_3 formation from the uncatalyzed hydration of CO_2.[2]

[2] In a cyclic process such as this the rate at which H_2CO_3 must be produced from CO_2, in order to accomplish the observed rates of HCO_3^- reabsorption, needs only be as great as the rate at which H_2CO_3 is lost from the cycle by decomposition in the tubular lumen. If, therefore, only 75 per cent of the luminal H_2CO_3 returned to the cell by diffusion and solvent drag and the remaining 25 per cent decomposed to CO_2 and H_2O, then only 25 per cent of the H^+ mediating HCO_3^- reabsorption need be produced from CO_2. Recycling of this magnitude could readily account for the calculated three- to fivefold deficit in H^+ production from CO_2.

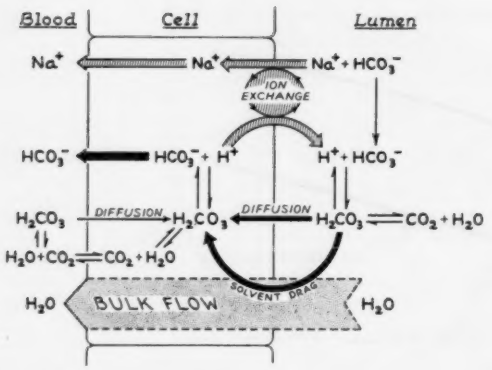

FIG. 7. ROLE OF BACK-DIFFUSION OF H_2CO_3 IN THE SUPPLY OF INTRACELLULAR H^+. By recycling into the cell, H_2CO_3 can furnish H^+ to the exchange process, and to that extent reduce the requirements for CO_2 hydration. The pCO_2 will nevertheless determine the steady state concentration of H_2CO_3 at which the system operates.

The capacity to reabsorb HCO_3^- in the absence of carbonic anhydrase activity increases as pCO_2 is elevated, but not in linear fashion (Figure 6, lower curve). The curvilinear character of this relationship could be due to a nonlinear relationship between: *1*) pCO_2 and H^+ production; *2*) H^+ production and intracellular H^+ concentration (resulting from more effective intracellular buffering at higher CO_2 tensions); or *3*) intracellular H^+ concentration and H^+ transport (progressive saturation of H^+ transport). The first two possibilities seem unlikely, since the production of H^+ via the uncatalyzed hydration of CO_2 is linearly related to pCO_2 *in vitro* (5), and since the titration of a thick homogenate of kidney with CO_2 showed that H^+ concentration of the homogenate was linearly related to pCO_2 (7). The curvilinear shape of the lower curve, therefore, probably reflects the kinetic characteristics of the H^+ transport mechanism as it becomes saturated with H^+. Plotting the reciprocal of HCO_3^- reabsorption against the reciprocal of plasma pCO_2 (Lineweaver-Burke plot) generates a straight line when carbonic anhydrase is inhibited (Figure 8). While the straight-line relationship does not permit identification of the specific process involved, it does suggest that the characteristics of this reabsorptive process are determined by a single rate-limiting step. A similar plot of data obtained from animals with normal carbonic anhydrase activity did not give a linear relationship (Figure 8). It would appear that HCO_3^- reabsorption in the presence of an intact carbonic anhydrase enzyme system is qualitatively as well as quantitatively different from HCO_3^- reabsorption in the absence of carbonic anhydrase.

In contrast to the uncatalyzed reaction, the reabsorption of that fraction of HCO_3^- mediated by carbonic anhydrase is strikingly independent of variations in pCO_2 (Figure 6). One possible explanation for this apparent insensitivity to changes in pCO_2 is that carbonic anhydrase becomes completely saturated with CO_2 at very low tensions (< 10 mm Hg) and thereafter contributes a constant quantity of H^+ to the reabsorptive process. This, however, would imply a Km for renal carbonic anhydrase of approximately 1×10^{-4} M, a value at great variance with the Km at 38° C of 760×10^{-4} M for purified red cell enzyme (15). From this latter Km it can be estimated that at plasma CO_2 tensions as high as 2,500 mm Hg carbonic anhydrase would be only half-saturated. To attribute the constant contribution of the carbonic anhydrase enzyme system to HCO_3^- reabsorption to early saturation of the enzyme would therefore appear to be untenable.

Actually, there is reason to believe that carbonic anhydrase is not simply contributing a constant quantity of H^+ in excess of that supplied by the uncatalyzed hydration of CO_2. Because the curve relating HCO_3^- reabsorption to pCO_2 in the absence of carbonic anhydrase flattens at high plasma CO_2 tensions, it was concluded that the H^+ transport mechanism was becoming saturated by an excess of H^+ ions. Increasing or decreasing the supply of H^+ in the range of H^+ excess should have little or no effect on HCO_3^- reabsorption. If carbonic anhydrase were simply contributing a fixed quantity of H^+ to the transport mechanism the two curves (Figure 6) should converge at higher CO_2 tensions, rather than remain parallel. The contribution of carbonic anhydrase, therefore, does not appear to be a simple addition of a constant quantity of H^+ to the same transport mechanism supplied by the uncatalyzed hydration of CO_2.

From this analysis of the effect of pCO_2 on the maximal HCO_3^- reabsorptive capacity, the HCO_3^- Tm can be characterized as the sum of two inde-

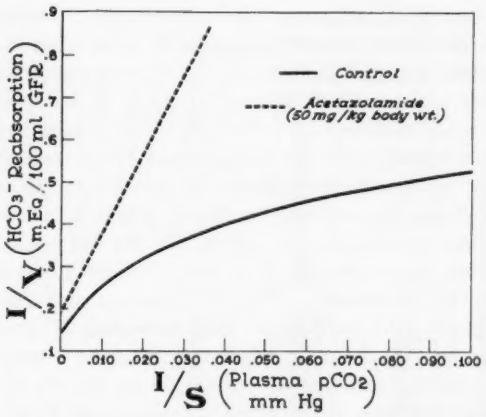

FIG. 8. DOUBLE RECIPROCAL PLOT OF THE RELATION BETWEEN HCO_3^- REABSORPTION AND PLASMA pCO_2 WITH AND WITHOUT ACETAZOLAMIDE. The solid line is the mean curve obtained by a double reciprocal of data from control animals (taken from Figure 1) and the broken line is the mean curve obtained by a similar plot of data from animals given acetazolamide (taken from Figure 2).

pendent reactions. One reaction is catalyzed by carbonic anhydrase, the other is uncatalyzed. As pCO_2 is increased the Tm increases, but solely as the result of an increase in the capacity of the uncatalyzed reaction, the capacity of the catalyzed reaction remaining constant. The regulation of HCO_3^- reabsorption in respiratory acidosis and alkalosis, therefore, is entirely independent of the carbonic anhydrase enzyme system.

Two mechanisms with such diverse reabsorptive properties suggest the existence of two different transport systems, one pCO_2-insensitive, the other pCO_2-sensitive. The fact that acetazolamide depresses the HCO_3^- Tm by a constant amount at all CO_2 tensions indicates that one transport system (pCO_2-insensitive) has a fixed capacity which is critically dependent upon the activity of carbonic anhydrase. Since changes in plasma pCO_2 have no effect on the capacity of this system, the H^+ transport mechanism must be saturated with H^+ and consequently insensitive to the changes in intracellular pH produced by variations in CO_2 tension. The dependence on carbonic anhydrase activity could result from the requirement of the transport system for a very rapid rate of H^+ supply (such as might occur if the ratio of transport capacity to cell volume were very high). A second transport system located elsewhere in the nephron is sensitive to changes in plasma pCO_2, suggesting that, in contrast to the pCO_2-insensitive mechanisms, it is unsaturated with respect to H^+ and therefore responsive to changes in intracellular pH. This system, which functions well in the absence of carbonic anhydrase, can be adequately supplied with H^+ by the uncatalyzed hydration of CO^+ supplemented by the recycling of H_2CO_3. Inhibition of carbonic anhydrase, therefore, has little effect on the reabsorptive capacity of this pCO_2-sensitive system.[3]

To characterize the two distinct mechanisms involved in HCO_3^- reabsorption from the vantage point of parameters other than maximal reabsorptive capacity, the relationship of HCO_3^- excretion to HCO_3^- reabsorption was studied when plasma HCO_3^- was maintained at high levels and carbonic anhydrase uninhibited (Figure 4, upper curve); HCO_3^- excretion began before the Tm was reached (magnitude of HCO_3^- leak indicated by stippled area). Schwartz, Falbriard and Lemieux (6), in similar experiments, found that in respiratory acidosis as plasma HCO_3^- concentration was increased from 26 to 55 mEq per L there was a curvilinear rise in HCO_3^- reabsorption without a definite Tm being attained. A double reciprocal plot (Lineweaver-Burke) of their data generated a straight line, which was interpreted as evidence that the high CO_2 tension had in some fashion altered the H^+ transport mechanism so that carbonic anhydrase had become the rate-limiting step in HCO_3^- reabsorption. In contrast, when the plasma HCO_3^- concentrations were increased over a far greater range in the present investigations, a Tm was clearly obtained in respiratory acidosis, with HCO_3^- reabsorption remaining constant as plasma HCO_3^- was increased from 42 to 80 mEq per L. The fact that a Tm was reached means that a double reciprocal plot would not generate a straight line. Consequently, this type of curvilinear relationship cannot be used as evidence that renal carbonic anhydrase is rate-limiting and, therefore, responsible for the shape of the curve.

[3] This does not imply that the cells involved in this transport process contain no carbonic anhydrase. Evidence will be presented later indicating that carbonic anhydrase may be present on the luminal border of the renal tubular cells. Its action, however, would not influence the HCO_3^- Tm.

TABLE V

Ability of HCO_3^- reabsorptive mechanism to effect complete HCO_3^- reabsorption (100% of filtered HCO_3^-) as the maximal reabsorptive capacity (Tm) is approached

HCO_3^- reab. / HCO_3^- Tm ×100	Filtered HCO_3^- reabsorbed		
	Normal pCO_2	Elevated pCO_2	Acetazolamide
%	%	%	%
50	100	100	65
70	99	100	60
75	99	99	59
80	98	98	58
85	95	96	56
90	92	92	55
95	90	90	50
100	83	83	42

When carbonic anhydrase was uninhibited and the pCO_2 maintained at normal values (Figure 4, middle curve), a distinct HCO_3^- leak was also demonstrated, HCO_3^- excretion commencing when plasma HCO_3^- concentration approached 18 mEq per L. This HCO_3^- leak does not appear to differ in any way from that observed during respiratory acidosis. It would appear that a HCO_3^- leak in each instance supervenes because the reabsorptive mechanism, when functioning at near-capacity levels, cannot effect complete HCO_3^- reabsorption, but can reabsorb some fixed percentage of the filtered HCO_3^- (possibly because of sensitivity to luminal pH when transporting H^+ at near-capacity rates). Thus, bicarbonate excretion begins in both the normal state and respiratory acidosis when the transport system is functioning at approximately 75 per cent of capacity (Table V). As reabsorption approaches 100 per cent of capacity the percentage of filtered HCO_3^- that is reabsorbed falls to approximately 83. The relationship between the percentage of capacity utilized and percentage of filtered HCO_3^- reabsorbed is identical in the normal state and respiratory acidosis. Although the magnitude of the HCO_3^- leak, in absolute quantities, is higher in respiratory acidosis, this is the consequence of the greater filtered HCO_3^- loads rather than a direct effect of pCO_2 on the characteristics of the transport mechanism.

The nature of the HCO_3^- leak following the administration of acetazolamide, however, appears to be quite different, qualitatively as well as quantitatively, from that observed in the normal state and in respiratory acidosis. It cannot be attributed to a simple exaggeration of the normal characteristics of H^+ secretion. It is evident from Figure 4 and Table V that inhibition of carbonic anhydrase impairs the ability of the reabsorptive process to produce HCO_3^--free urines at all levels of HCO_3^- reabsorption. This large HCO_3^- leak does not appear to be due to decreased H^+ production alone, since raising plasma CO_2 tension to 110 to 130 mm Hg restored the HCO_3^- Tm to a normal value of 2.7 mEq per 100 ml GF but did not reduce the magnitude of the leak (Figure 5, upper curve). Recently Schwartz, Lemieux and Falbriard (16) have shown that decreasing H^+ production by means of respiratory alkalosis without inhibiting carbonic anhydrase lowers the HCO_3^- Tm but does not produce a comparable HCO_3^- leak. It seems unlikely, therefore, that diminished H^+ production could account for the HCO_3^- leak.

To test more rigorously whether acetazolamide might augment the HCO_3^- leak by eliminating H^+ production via carbonic anhydrase, the capacity of the uncatalyzed reaction was increased by elevating plasma pCO_2 under circumstances where filtered HCO_3^- was comparatively low. The data from Table IV are schematically presented in Figure 9. The value of the maximal capacity of the the uncatalyzed reaction (black bar) at each pCO_2 was obtained from the lower curve in Figure 6 and serves as a basis for comparison with the observed HCO_3^- reabsorption (clear bar). Despite the fact that, as pCO_2 was raised, the capacity of the uncatalyzed reaction always exceeded the filtered HCO_3^- (cross-hatched bar) by a considerable amount, HCO_3^- reabsorption remained incomplete and HCO_3^- excretion continued. The enhanced HCO_3^- leak following the administration of acetazolamide, therefore, cannot be the consequence of deficient H^+ production.

The most likely explanation for the singular importance of carbonic anhydrase in maintaining the ability of the H^+ transport system to render the urine HCO_3^--free hinges about its possible luminal action (17). If, in addition to its distribution in the cytoplasm of renal tubular cells, the enzyme were also present on the luminal border of the cells, H_2CO_3 could not accumulate in luminal fluid. As a consequence, tubular pH would not fall to limiting values until $NaHCO_3$ reabsorption was virtually complete. However, when carbonic anhydrase was inhibited, H_2CO_3 would accumulate in the tu-

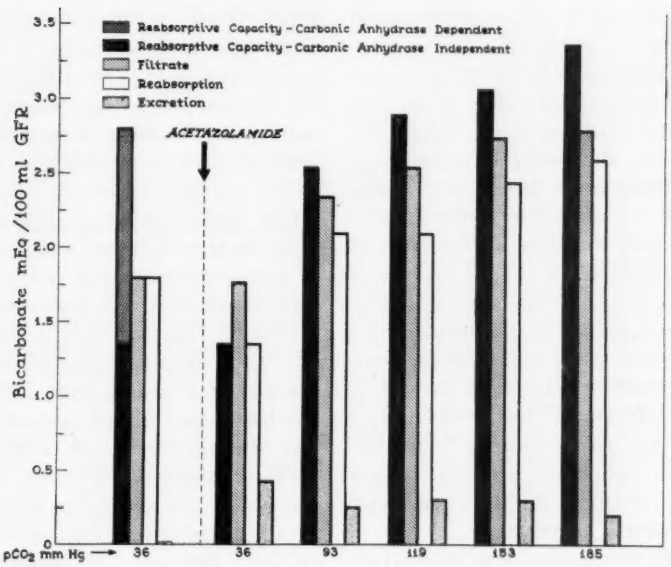

FIG. 9. FAILURE OF INCREASING PLASMA pCO_2 TO OBLITERATE BICARBONATE EXCRETION IN THE ABSENCE OF CARBONIC ANHYDRASE. See text for explanation.

bular lumen, lowering luminal pH to a limiting value despite the presence of significant amounts of $NaHCO_3$.[4] As a result of the low tubular pH, net H^+ secretion would stop, thereby preventing the complete removal of HCO_3^- from the urine.

The repression of net H^+ secretion resulting from inhibition of the luminal action of carbonic anhydrase could be eventually overcome, however, by raising the concentration of HCO_3^- in glomerular filtrate. As HCO_3^- concentration is increased, the ionization of the accumulated H_2CO_3 is repressed, permitting further H^+ secretion, until finally the maximal reabsorptive capacity is realized.[5] Inhibition of the luminal action of carbonic anhydrase, therefore, would always augment a HCO_3^- leak but would not alter the HCO_3^- Tm.

The inability to produce HCO_3^--free urines in the absence of carbonic anhydrase suggests that the pCO_2-sensitive system, whose capacity is independent of an intracellular action of carbonic anhydrase, is sensitive to luminal pH and, therefore, dependent upon the luminal action of carbonic anhydrase. Since high urinary CO_2 tensions have been observed duing HCO_3^- diuresis produced by the administration of $NaHCO_3$ in the presence of an intact carbonic anhydrase enzyme system (11), it is unlikely that carbonic anhydrase exerts a luminal action in the distal tubule. It seems probable, therefore, that the pCO_2-sensitive system, which is dependent upon the luminal action of carbonic anhydrase, is located in the more proximal portions of the nephron.

To explain the results of the present studies it is proposed that HCO_3^- reabsorption is mediated by two distinct H^+ secretory systems, one located in the proximal tubule, the other in the distal tubule. The proximal tubular mechanism appears to be sensitive to changes in intracellular as well as luminal pH. Alterations in plasma CO_2 tension, by changing the concentration of H_2CO_3 in the cell, elicit prompt changes in H^+ secretion.

[4] Under such circumstances, therefore, although bladder urine would be alkaline, the tubular fluid would be acid because of the accumulation of H_2CO_3. The high pCO_2 of such bladder urine indicates that in the tubular lumen, H_2CO_3 must have been present in significant amounts.

[5] In this manner filtered HCO_3^- would, in a sense, be competing with cellular processes for available H^+ and, as previously suggested (4), result in a curvilinear relationship between HCO_3^- reabsorption and plasma HCO_3^- concentration.

The sensitivity to luminal pH prevents transport of H^+ against sharp pH gradients. Carbonic anhydrase, by catalyzing the dehydration of H_2CO_3 as it is formed at the luminal surface of the cell, minimizes acidification of the urine, thereby facilitating continued H^+ transport; the enzyme may also be located within the cell, thereby augmenting H_2CO_3 production. Inhibition of carbonic anhydrase would diminish the rate of formation of H_2CO_3 from the hydration of CO_2 as a result of its intracellular action, and at the same time cause the accumulation of H_2CO_3 in the tubular fluid because of its luminal action. The latter effect, by enhancing the return of H_2CO_3 to the cell by a process of back-diffusion and solvent drag, could maintain H^+ transport despite reduced H^+ production. The HCO_3^- Tm of the proximal tubular transport system, therefore, would be unaffected by inhibition of carbonic anhydrase.

The distal tubular transport system, by contrast, is relatively insensitive to both intracellular and luminal pH. Consequently, the secretion of H^+ is neither influenced by alterations in plasma CO_2 tension nor dependent upon a luminal action of carbonic anhydrase. The capacity of this transport system is geared to a very rapid supply of H^+ and is, therefore, critically dependent upon the intracellular action of carbonic anhydrase.

Inhibition of carbonic anhydrase produces two distinct effects: *1*) a reduction in HCO_3^- Tm resulting primarily from diminution of distal tubular H^+ secretion, and *2*) an exaggerated HCO_3^- leak resulting from a combination of decreased distal tubular H^+ secretion and H_2CO_3 accumulation in the proximal tubular fluid (preventing thereby complete HCO_3^- reabsorption). Alterations in pCO_2, on the other hand, affect HCO_3^- reabsorption solely by changing the rate of H^+ secretion by the proximal tubular system.

SUMMARY

Renal HCO_3^- reabsorption was examined by three types of experiments. In the first group the effect of plasma pCO_2 on maximal HCO_3^- reabsorptive capacity (HCO_3^- Tm) was assessed in 15 dogs before and after inhibition of carbonic anhydrase. Bicarbonate reabsorption increased curvilinearly as plasma pCO_2 was elevated. Inhibition of carbonic anhydrase depressed the HCO_3^- Tm by a constant amount at all CO_2 tensions. From these studies it appeared that the contribution of carbonic anhydrase to the HCO_3^- Tm was completely independent of pCO_2, and that the regulatory effects of pCO_2 were mediated entirely through the uncatalyzed hydration of CO_2. In the absence of carbonic anhydrase activity all HCO_3^- reabsorption was dependent upon CO_2.

In the second group of experiments the effects of variations in plasma pCO_2 and inhibition of carbonic anhydrase on the excretion of HCO_3^- as the HCO_3^- Tm was approached were studied in 15 dogs. When plasma pCO_2 was maintained constant at a normal level, HCO_3^- excretion began before the Tm was reached. A similar leak was noted in respiratory acidosis. Carbonic anhydrase inhibition, however, caused a HCO_3^- leak which was of greater magnitude and which occurred even at very low concentrations of plasma HCO_3^-. Increasing H^+ production by elevating plasma pCO_2 to 110 to 130 mm Hg failed to obliterate the HCO_3^- leak.

In the third group (four dogs) elevating plasma pCO_2 as high as 200 mm Hg when carbonic anhydrase was inhibited did not significantly diminish HCO_3^- excretion despite marked reductions in the filtered HCO_3^- load as a result of metabolic acidosis.

It was concluded that HCO_3^- reabsorption was accomplished by two distinct processes. One process, presumably located in the proximal tubule, has a HCO_3^- Tm which is dependent upon plasma CO_2 tension and independent of carbonic anhydrase, and a transport system sensitive to the pH of tubular fluid. Carbonic anhydrase, by catalyzing the dehydration of carbonic acid at the luminal surface, prevents drastic lowering of the pH, thereby facilitating HCO_3^- reabsorption. A second process, apparently located in the distal tubule, has a fixed HCO_3^- Tm which is dependent upon carbonic anhydrase, independent of changes in plasma pCO_2 and can operate efficiently despite sharp pH gradients.

REFERENCES

1. Brazeau, P., and Gilman, A. Effect of plasma CO_2 tension on renal tubular reabsorption of bicarbonate. Amer. J. Physiol. 1953, **175**, 33.
2. Relman, A. S., Etsten, B., and Schwartz, W. B. The regulation of renal bicarbonate reabsorption by

plasma carbon dioxide tension. J. clin. Invest. 1953, **32**, 972.

3. Dorman, P. J., Sullivan, W. J., and Pitts, R. F. The renal response to acute respiratory acidosis. J. clin. Invest. 1954, **33**, 82.
4. Seldin, D. W., Portwood, R. M., Rector, F. C., Jr., and Cade, R. Characteristics of renal bicarbonate reabsorption in man. J. clin. Invest. 1959, **38**, 1663.
5. Davis, R. P. The kinetics of the reaction of human erythrocyte carbonic anhydrase. I. Basic mechanism and the effect of electrolytes on enzyme activity. J. Amer. chem. Soc. 1958, **80**, 5209.
6. Schwartz, W. B., Falbriard, A., and Lemieux, G. The kinetics of bicarbonate reabsorption during acute respiratory acidosis. J. clin. Invest. 1959, **38**, 939.
7. Rector, F. C., Jr. Unpublished observations.
8. Maren, T. H., Tarcell, A. L., and Malik, M. N. A kinetic analysis of carbonic anhydrase inhibition. J. Pharmacol. exp. Ther. In press.
9. Maren, T. H., Wadsworth, B. C., Yale, E. K., and Alonso, L. G. Carbonic anhydrase inhibition III. Effects of Diamox on electrolyte metabolism. Johns Hopk. Hosp. Bull. 1954, **95**, 277.
10. Schwartz, W. B., Falbriard, A., and Relman, A. S. An analysis of bicarbonate reabsorption during partial inhibition of carbonic anhydrase. J. clin. Invest. 1958, **37**, 744.
11. Pitts, R. F., and Alexander, R. S. The nature of the renal tubular mechanism for acidifying the urine. Amer. J. Physiol. 1945, **144**, 239.
12. Berliner, R. W. Renal secretion of potassium and hydrogen ions. Fed. Proc. 1952, **11**, 695.
13. Davies, R. E. Hydrochloric acid production by isolated gastric mucosa. Biochem. J. 1948, **42**, 609.
14. Roughton, F. J. W., and Clark, A. M. Carbonic anhydrase *in* The Enzymes, J. B. Sumner and K. Myrbäck, Eds. New York, Academic Press, 1951, vol. 1, pt. 2, pp. 1250–1265.
15. Kiese, M. Kinetik der Kohlensäureanhydrase. I. Biochem. Z. 1941, **307**, 400.
16. Schwartz, W. B., Lemieux, G., and Falbriard, A. Renal reabsorption of bicarbonate during acute respiratory alkalosis. J. clin. Invest. 1959, **38**, 2197.
17. Walser, M., and Mudge, G. H. Renal excretory mechanisms *in* Mineral Metabolism, C. L. Comar and F. Bronner, Eds. New York, Academic Press, 1960.

CIRCULATORY EFFECTS OF CHRONIC HYPERVOLEMIA IN POLYCYTHEMIA VERA*

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In this study the relationship of cardiac output to oxygen supply on the one hand and to blood volume on the other is examined in patients with polycythemia vera.

Oxygen supply is a function of the cardiac output and the oxygen content of arterial blood. There is good evidence that cardiac output may be altered by changes in either tissue oxygen requirements or oxygen content. Thus, with increased physical activity or hyperthyroidism, cardiac output is elevated. Likewise, in severe anemia or with the hypoxia of high altitude, cardiac output rises. The relationship between cardiac output and arterial oxygen content was well illustrated by Richardson and Guyton in dogs whose hematocrits were rapidly altered by blood exchange without blood volume change (1). Cardiac output fell with polycythemia and rose with anemia; maximal oxygen delivery occurred at a normal hematocrit. From the above considerations it would seem reasonable to postulate that cardiac output is regulated in some manner by tissue oxygen requirements and blood oxygen concentration.

The influence of blood volume as a cardiac regulatory mechanism has been demonstrated in dogs as well as in man (2–6). Such observations following the acute manipulation of blood volume have shown an augmentation of cardiac output and stroke volume in hypervolemia. The effects of chronic hypervolemia in cardiovascular regulation have not been reported.

Polycythemia vera presents an interesting situation in which the above factors may be examined. There is a long-standing surplus of hemoglobin and blood oxygen without a proportionate increase in tissue oxygen requirements; furthermore, blood volume is increased in polycythemia vera. The increased arterial oxygen content and blood viscosity might be expected to decrease cardiac output, whereas chronic hypervolemia might have the opposite effect. Our studies were designed to evaluate these possibilities.

MATERIAL AND METHODS

Three types of subjects were selected for study. Group Ia consisted of 6 subjects with incidental illness not considered to affect erythropoiesis or cardiac function. These individuals were ambulatory and asymptomatic; they had hematocrits between 40 and 50 per cent and normal blood volumes. There was no recognizable cardiac or pulmonary disease. Electrocardiograms were all normal, and heart size by X-ray was not enlarged. Group Ib consisted of 4 subjects with hematocrits of 50 to 55 per cent and with normal or only slightly enlarged blood volumes. These subjects also had no evidence of cardiac or pulmonary disease. X-rays showed normal sized hearts and, except for isolated left axis deviation in D.G., electrocardiograms were normal.

Group II consisted of 10 patients with polycythemia vera, 5 of whom had symptoms of approximately 1 month's duration. The other 5 had been previously treated, but were all polycythemic and hypervolemic at the time of initial studies. The spleen was palpable in all subjects except S.Ta., S.Tu. and B.P. Electrocardiograms were normal, except for left ventricular hypertrophy in Patient S.Ta. and right bundle branch block in B.P. Patient A.L. had a previous history of paroxysmal auricular fibrillation. Cardiac enlargement of 25, 20 and 25 per cent was present in Patients S.Ta., G.S. and A.L., but was normal in the other subjects (± 10 per cent). Pertinent clinical data on the 20 subjects studied are summarized in Tables I and II.

Single studies were performed in Groups Ia and Ib. In 7 patients with polycythemia vera, studies were repeated after phlebotomy. The patients were in the supine position when the hemodynamic measurements were made 1 to 3 hours after a light nonfat breakfast. No premedication was given. Intravascular pressures were recorded on a Sanborn polygraph using Statham P23D strain gage transducers. Heart rate was measured from the electrocardiogram obtained during the determination of cardiac output. Cardiac output was measured by the

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TABLE I

Clinical and laboratory summary of normovolemic subjects

Patient	Age	Sex	Weight	Mean brachial arterial pressure	Arterial hematocrit	Arterial oxygen % saturation	
						Air	100 % Oxygen
	yrs		*kg*	*mm Hg*	%		
Ia Normal hematocrit							
A.R.	42	M	59.5	82	0.43	95.2	
D.B.	49	M	86.4	103	0.48	95.8	
W.S.	50	M	82.6	103	0.41		
C.H.	71	F	69.0	99	0.49	97.3	
Z.B.	52	F	64.8	100	0.44	96.5	
O.W.	42	F	66.6	104	0.40	95.3	
Ib Elevated hematocrit							
G.A.	47	M	86.5	82	0.53	98.9	100^{-}
D.G.	73	F	55.7	116	0.53	96.0	100^{+}
C.D.	44	M	70.5	94	0.53	94.2	99.2
F.B.	65	M	57.5	90	0.55		

direct Fick principle in one patient (S.Tu.) and with indicator dilution curves in the others. Mixed venous blood was sampled from the pulmonary artery and arterial blood from the brachial artery. Oxygen consumption was measured with a 9 L Collins respirometer while the subjects were breathing 100 per cent oxygen. Oxygen content of blood was determined by the Van Slyke-Neill method.

Indicator dilution curves were obtained while the subjects were breathing 100 per cent oxygen from the Collins respirometer. Thirteen to 19 mg of Evans blue dye was rapidly injected from a calibrated syringe into the main pulmonary artery in 3 patients (D.G., G.S. and A.L.) and into an antecubital vein, with prompt elevation of the subject's arm, in the remaining 17. Using a rotating multiple-sampling device which was adjusted to collect samples at 2-second intervals, brachial arterial blood was collected through a no. 17-T Cournand needle and a short polyethylene tube, in tubes containing a few particles of powdered heparin. The arterial blood was centrifuged, and the optical density of the undiluted plasma was read twice with a Beckman DU spectrophotometer at 620 mμ. Indicator dilution curves were constructed on semilogarithmic paper from the plasma concentration of T-1824. Cardiac output and mean transit time were calculated by mathematically extrapolating the downslope of the dye curve to zero concentration as described by Lilienfield and Kovach (7). Stroke volume was obtained by dividing cardiac output by heart rate. Arterial hematocrits were read in Wintrobe tubes after centrifugation at 2,260 G for 30 minutes. Red cell volume was measured by the dilution of Cr^{51}-labeled red cells as described previously (8). In calculating plasma volume from the Cr^{51} cell determinations, the total body hematocrit was assumed to be 0.92 times the large vessel hematocrit (8, 9). Direct measurement from T-1824 concentrations after 10 minutes of equilibration gave similar plasma volume measurements. The blood volume data recorded in Table III are those derived from the Cr^{51} method.

RESULTS

Data obtained in individual subjects are summarized in Table III. Oxygen consumption ap-

TABLE II

Clinical and laboratory summary of subjects with chronic hypervolemia (polycythemia vera)

Patient	Age	Sex	Weight	Duration of symptoms	Previous treatment	Arterial Hct.	Mean brachial arterial pressure	WBC	Platelets	Arterial oxygen % saturation	
										Air	100% Oxygen
	yrs		*kg*	*mos*		%	*mm Hg*	*10^3/cu mm*			
S.Ta.	76	F	45.3	1	0	0.65	110	10.9	186	95.0	100^{+}
G.S.	65	F	51.4	1	0	0.65	120	16.8	850	93.0	99.5
A.L.	61	M	71.8	1	0	0.56	120	15.3	830	94.1	98.7
F.V.	42	M	67.3	1	0	0.60	97	22.9	1,315	94.0	97.4
T.S.	68	M	58.0	1	0	0.65	128	18.0	510	93.3	100^{+}
H.W.	68	M	63.6	12	P^{32}	0.59	99	10.3	220	93.7	99.5
R.S.	55	M	69.0	12	P^{32}	0.56	108	13.3	330	97.0	100^{+}
S.Tu.	33	F	58.0	156	*	0.57	81	8.4	220	99.5	100^{+}
J.F.	33	M	75.9	60	P^{32}	0.64	100	22.8	258	96.9	99.0
B.P.	65	M	66.9	84	P^{32}	0.56	100	10.8	184	91.6	100^{+}

* One or more phlebotomies.

TABLE III

Resting hemodynamics in normovolemic and hypervolemic subjects

Subject*	BSA	RBC vol.	Total blood vol.	Arterial hct.	Cardiac index	Plasma flow	Heart rate	Stroke index	Mean transit time†	Oxygen consumption	Interval between studies	Amount blood removed
	m²	*ml/kg*	*ml/kg*	*%*	*L/min/m²*		*beats/min*	*ml/beat/m²*	*sec*	*ml/min/m²*	*days*	*L*
Group Ia												
A.R.	1.69	22.9	57.5	0.43	4.18	2.38	93	45	17.8			
D.B.	2.16	30.6	70.0	0.48	3.46	1.82	80	43	19.5	136		
W.S.	2.09	26.6	71.9	0.41	4.31	2.56	89	48	17.0			
C.H.	1.69	27.4	60.6	0.49	3.56	1.82	83	43	20.3	170		
Z.B.	1.68	18.9	46.6	0.44	2.81	1.57	79	36	16.4	152		
O.W.	1.68	20.3	55.0	0.40	3.60	2.16	85	42	14.6	104		
Group Ib												
G.A.	2.10	29.9	61.5	0.53	3.79	1.78	83	46	18.7	200		
D.G.	1.53	32.3	66.8	0.53	3.26	1.55	84	39	11.5†	119		
C.D.	1.81	33.5	68.4	0.53	3.61	1.71	86	42	17.9	162		
F.B.	1.67	41.3	75.1	0.55	3.35	1.51	87	39	15.9	137		
Group II												
S.Ta. b	1.46	55.7	93.2	0.65	5.61	1.96	110	51		136		
a	1.46	37.6	85.2	0.48	3.83	1.99	101	38		134	15	1.5
G.S. b	1.43	62.3	104.2	0.65	5.45	1.91	80	68	12.0†	168		
a	1.43	40.1	97.3	0.45	4.87	2.68	77	63	13.2†	204	23	2.0
A.L. b	1.83	62.0	120.0	0.56	5.99	2.64	91	66	9.7†	178		
a	1.94	30.4	87.3	0.38	5.19	3.22	90	58	7.6†	193	62	3.1
F.Y. b	1.85	54.1	94.6	0.60	4.11	1.64	70	59	28.8			
a	1.88	39.4	85.8	0.46	3.32	1.79	69	48	24.6		37	1.0
T.S. b	1.68	56.5	94.5	0.65	4.14	1.45	72	58	21.8	142		
a	1.68	34.8	79.8	0.48	3.94	2.05	69	57	18.1	152	21	2.4
H.W. b	1.71	50.3	94.8	0.59	3.60	1.48	57	63	26.3	143		
a	1.71	34.8	82.1	0.46	3.36	1.81	62	54	25.7	194	14	1.5
R.S. b	1.75	39.3	80.9	0.56	5.31	2.34	108	49	14.6	153		
a	1.75			0.55	4.51	2.03	107	42	17.5	150	<1	0.5
S.Tu.	1.62	43.8	83.6	0.57	3.77	1.62	71	53	18.8	128		
J.F.	1.97	65.0	110.5	0.64	6.14	2.21	88	70	18.8	188		
B.P.	1.84	38.9	75.5	0.56	3.97	1.75	77	52	18.8			

* b, Before blood removal; a, after blood removal.
† Antecubital vein to brachial artery except in D.G., G.S. and A.L. where injection site was main pulmonary artery.

peared to be similar between the subjects in Groups I and II. The average for the former was 148 ml per m^2 per minute, and in subjects with polycythemia vera, 155 ml per m^2 per minute. The mean arterial oxygen saturations were 96, 96 and 95 per cent in Groups Ia, Ib and II. Oxygen content of blood differed; Group Ia had an average arterial oxygen content of 17.83; Group Ib, 20.81; Group II, 22.04 volumes per cent.

The average cardiac output of 3.65 L per minute per m^2 in the normovolemic subjects with normal hematocrit (Group Ia) was similar to that of 3.50 L per minute per m^2 in Group Ib with elevated hematocrits but normal blood volume. In the polycythemia group (II), output was 32 per cent higher, i.e., 4.81 L per minute per m^2. When both arterial oxygen content and cardiac output are considered, the total oxygen available to the tissues per unit of time was greater by 19 per cent in Group Ib and by 70 per cent in Group II, compared with Group Ia.

Blood volume measurements indicated an average red cell mass of 24 ml per kg in Group Ia, 34 ml per kg in Group Ib, and 53 ml per kg in Group II. Plasma volumes were 36, 34 and 42; total blood volumes were 60, 68 and 95 ml per kg. The body hematocrit correction factor [1] was 0.87, 0.92, and 0.91 in Groups Ia, Ib, and II. These similar values would seem to exclude any significant blood vessel distributional difference among the three groups. Five of the 10 hypervolemic patients with polycythemia vera had high cardiac outputs (Figure 1). Comparing those with high and normal output, the former had an average blood volume of 102 ml per kg versus 89 ml per kg in the latter. There was no apparent difference between the five subjects with normal output and the five with high output when evaluated from the standpoint of duration of illness, splenomegaly, hematocrit level, oxygen consumption or age. Pulse rate was greater in the subjects with a high output (95 versus 69 beats per minute). There was a high degree of correlation (R = + 0.94) between blood volume and resting stroke volume in all subjects studied. The output per beat per m^2

[1] Where mean body hematocrit correction factor = $\frac{\text{red cell volume } (Cr^{51})}{\text{red cell volume } (Cr^{51}) + \text{plasma volume T-1824}} \times \frac{1}{\text{arterial hematocrit}}$

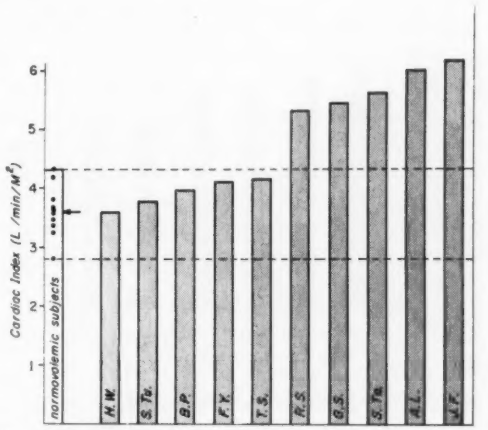

FIG. 1. RESTING CARDIAC OUTPUT EXPRESSED AS CARDIAC INDEX IN CONTROLS AND POLYCYTHEMIC SUBJECTS.

exceeded normal values in all of the polycythemia patients, and the correlation coefficient between blood volume and stroke volume corrected for body surface area was + 0.91 (Figure 2). Similarly, cardiac index correlated well with blood volume per m^2 (R = + 0.76). The mean transit time (antecubital vein to brachial artery) of 21.1 seconds in the hypervolemic patients was slightly greater than the average value of 17.6 seconds in the normovolemic subjects.

These results suggested a relationship between the increased blood volume and elevated stroke output. In order to evaluate this relationship and the significance of the other measurements, seven patients with polycythemia vera were restudied following phlebotomy. Between 500

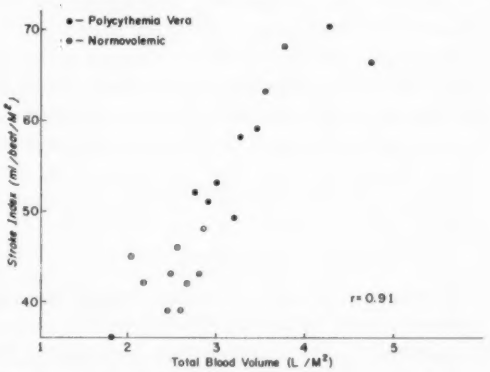

FIG. 2. THE RELATIONSHIP BETWEEN TOTAL BLOOD VOLUME AND RESTING STROKE INDEX IN NORMOVOLEMIC AND POLYCYTHEMIC SUBJECTS.

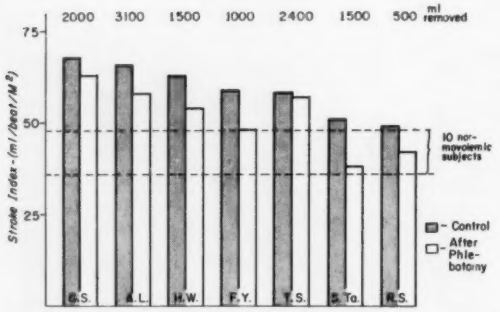

FIG. 3. THE EFFECT OF BLOOD REMOVAL ON STROKE INDEX IN POLYCYTHEMIA VERA.

and 3,100 ml of blood was removed from the patients over time intervals of up to 62 days. In the six subjects studied two to eight weeks post-phlebotomy (excluding the one instance when measurements were made immediately after bleeding), the average hematocrit was reduced from 0.62 to 0.45. Blood volume fell from 100.2 to 86.3 ml per kg. Cardiac index was reduced from 4.8 to 4.1 L per minute per m^2, and stroke index fell correspondingly from 61 to 53 ml per beat per m^2. The decreases, while not of great magnitude, were significant in that they occurred in each patient subjected to phlebotomy (Figure 3).

In the instances of intracardiac pressures measured in polycythemia vera (S.Ta., G.S. and A.L.), mean right atrial pressures were 1, 3 and 3; mean pulmonary artery, 12, 13 and 25; and pulmonary "capillary," 9, 9 and 11 mm Hg.

In the patients with polycythemia there was a significant correlation of total blood volume and oxygen consumption per m^2; the coefficient of correlation between these parameters prior to phlebotomy was 0.89 ($p < 0.001$). Oxygen consumption in the normovolemic subjects, however, showed no such relationship, although there was considerable overlap in oxygen consumption between the two groups. Red cell mass in the polycythemic subjects was similarly related to oxygen uptake.

DISCUSSION

Previous estimates of cardiac output in polycythemia vera have been limited and have been performed by the indirect Fick principle. Altschule, Volk and Henstell, employing the ethyl iodide method of measurement, reported normal output in three patients (10). Brooks also reported a normal cardiac index in one patient (11). Several others have agreed that retardation of peripheral blood flow is a prominent feature of polycythemia vera (12). Goldsmith, estimating output by the acetylene method, reported increased output in one of four patients studied (13). These reports are so limited in number as well as in methods that conclusions can not be drawn.

The present studies show a normal or elevated cardiac output and a consistently increased stroke volume in polycythemia vera. This is opposite to the experimental results obtained by Richardson and Guyton (1) in acutely induced normovolemic polycythemia.

The explanation for the elevated stroke index would appear to relate to the altered blood volume in polycythemia vera. That hypervolemia exerts its effect through increased central blood volume and enhanced diastolic filling seems logical (14). It is possible that the increased cardiac output observed in five of the ten subjects was in part dependent upon increased venous return, similar to the transient effects described with acute hypervolemia in dogs (15). From other studies there is evidence that acute hypervolemia achieved by the infusion of dextran and other blood volume expanders increases output (2, 4, 6, 16). These techniques also produce a decrease in hematocrit as a complicating feature. The present study indicates that high output occurs with hypervolemia even in the presence of an elevated hematocrit. These observations in the chronic hypervolemia of polycythemia vera may be compared with the studies of Kjellberg, Rudhe and Sjöstrand in which an augmentation of blood volume and work capacity per heart beat were observed in trained athletes (17). The possible relationship between increased output observed with high altitude polycythemia (18) and altered blood volume would seem to merit study.

Although blood volume correlated with both stroke volume and cardiac output in this study, the question should be raised whether hypervolemia was a causal factor or whether this correlation was incidental. The fall in cardiac output and stroke volume after blood volume was reduced by phlebotomy suggests that the hyperkinemia does indeed causally relate to chronic hypervolemia or some feature thereof. Recent observations on normal subjects show that acute bleeding results

in a decreased stroke volume which is correlated with the reduction in "central blood volume" (19). It was of some interest also that the effect of the increased cardiac output was to maintain a normal plasma flow in the patients (Figure 4). Although body tissues may have plasma flow requirements that influence cardiac regulation, such considerations at present remain conjectural.

Under the conditions of this study there is a notable lack of correlation between oxygen requirement, as reflected by oxygen consumption, and oxygen supply, as measured by hemoglobin concentration and cardiac output. In looking further into possible correlations, it is equally apparent that poor correlation exists between oxygen consumption, which presents similar average values in the three groups of subjects, and the blood volume, which is considerably elevated in the polycythemia vera group. There is, however, an impressive correlation between blood volume and oxygen consumption among the individuals of the polycythemia vera group ($R = +0.89$). One might suggest that increased blood volume per se could affect cardiac work and perhaps metabolic activity of other tissues. There was some correlation between oxygen consumption and cardiac work ($R = +0.69$) and stroke volume ($R = +0.78$), but not with heart rate or arterial blood pressure. However, post-phlebotomy studies appear to negate this reasoning, since a reduction in blood volume was associated with no change, or perhaps with a slight increase in oxygen consumption. A second explanation would seem to be the possible contribution of enhanced hematopoietic activity to the general body metabolic rate. Thus the increased red cell mass or blood volume and the enhanced metabolic activity would both be manifestations of the marrow disturbance. The explanation for this association awaits further studies, but two additional features might be mentioned: *1*) that while Bliss (20) reported a tendency for increased metabolic rate in polycythemia vera, our average oxygen consumption is not above that of the two other groups studied; and *2*) while there is a correlation between oxygen consumption and total blood volume or red blood cell mass, there is no correlation between oxygen consumption and hematocrit levels in the polycythemia vera patients.

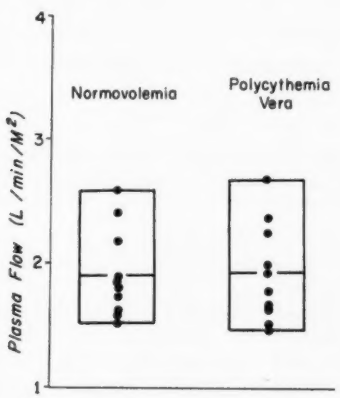

FIG. 4. PLASMA FLOW IN NORMOVOLEMIC AND POLYCYTHEMIC SUBJECTS.

SUMMARY AND CONCLUSIONS

Resting hemodynamic studies were performed in 10 patients with expanded blood volumes due to polycythemia vera. In comparison with comparable groups of normovolemic subjects, the patients with polycythemia showed a consistently elevated stroke index (39 per cent) as well as a significant but less uniform increase in cardiac index (34 per cent).

The augmentation of stroke volume described in these patients was closely related to the increase in total blood volume. There was excellent correlation between resting stroke index and total blood volume per m^2 in the 20 patients studied ($R = +0.91$).

Phlebotomies, performed in seven patients, were followed by a reduction in stroke volume and cardiac output in all instances.

The rate of plasma flow was found to be normal in polycythemia vera.

In patients with polycythemia there was a significant correlation between oxygen consumption and total blood volume.

ACKNOWLEDGMENTS

The authors are grateful to Doctors Daniel H. Coleman, Quin B. DeMarsh, Harold E. Laws and Alexander R. Stevens, Jr., for affording us the opportunity to study their patients.

REFERENCES

1. Richardson, T. Q., and Guyton, A. C. Effects of polycythemia and anemia on cardiac output and other circulatory factors. Amer. J. Physiol. 1959, **197**, 1167.

2. Witham, A. C., Fleming, J. W., and Bloom, W. L. The effect of the intravenous administration of dextran on cardiac output and other circulatory dynamics. J. clin. Invest. 1951, **30**, 897.
3. Ferguson, T. B., Shadle, O. W., and Gregg, D. E. Effect of blood and saline infusion on ventricular and diastolic pressure, stroke work, stroke volume and cardiac output in the open and closed chest dog. Circulat. Res. 1953, **1**, 62.
4. Gowdey, C. W., Hatcher, J. D., and Sunahara, F. A. Cardiovascular responses in dogs to large intravenous infusions. Canad. J. Biochem. 1954, **32**, 282.
5. Holt, J. P. Effect of plethora and hemorrhage on left ventricular volume and pressure. Circulat. Res. 1957, **5**, 273.
6. Schnabel, T. G., Jr., Eliasch, H., Thomasson, B., and Werkö, L. The effect of experimentally induced hypervolemia on cardiac function in normal subjects and patients with mitral stenosis. J. clin. Invest. 1959, **38**, 117.
7. Lilienfield, L. S., and Kovach, R. D. Simplified method for calculating flow, mean circulation time and downslope from indicator-dilution curves. Proc. Soc. exp. Biol. (N. Y.) 1956, **91**, 595.
8. Donohue, D. M., Motulsky, A. G., Giblett, E. R., Pirzio-Biroli, G., Viranuvatti, V., and Finch, C. A. The use of chromium as a red-cell tag. Brit. J. Haemat. 1955, **1**, 249.
9. Chaplin, H., Jr., Mollison, P. L., and Vetter, H. The body/venous hematocrit ratio: Its constancy over a wide hematocrit range. J. clin. Invest. 1953, **32**, 1309.
10. Altschule, M. D., Volk, M. C., and Henstell, H. Cardiac and respiratory function at rest in patients with uncomplicated polycythemia vera. Amer. J. med. Sci. 1940, **200**, 478.
11. Brooks, W. D. W. Circulatory adjustments in polycythemia rubra vera. Proc. roy. Soc. Med. 1936, **29**, 1379.
12. Lawrence, J. H. Polycythemia. New York, Grune and Stratton, 1955, p. 8.
13. Goldsmith, G. Cardiac output in polycythaemia vera. Arch. intern. Med. 1936, **58**, 1041.
14. Sjöstrand, T. Volume and distribution of blood and their significance in regulating the circulation. Physiol. Rev. 1953, **33**, 202.
15. Guyton, A. C., Lindsey, A. W., Kaufmann, B. N., and Abernathy, J. B. Effect of blood transfusion and hemorrhage on cardiac output and on the venous return curve. Amer. J. Physiol. 1958, **194**, 263.
16. Fowler, N. O., Franch, R. H., and Bloom, W. L. Hemodynamic effects of anemia with and without plasma volume expansion. Circulat. Res. 1956, **4**, 319.
17. Kjellberg, S. R., Rudhe, U., and Sjöstrand, T. Increase of the amount of hemoglobin and blood volume in connection with physical training. Acta physiol. scand. 1949, **19**, 146.
18. Theilen, E. O., Gregg, D. E., and Rotta, A. Exercise and cardiac work response at high altitude. Circulation 1955, **12**, 383.
19. Ralston, L. A., Cobb, L. A., and Bruce, R. A. The role of blood volume in regulation of cardiac output, the effects of arterial bleeding in normal subjects. Clin. Res. 1960, **8**, 124.
20. Bliss, T. L. Basal metabolism in polycythemia vera. Ann. intern. Med. 1929, **2**, 1155.

CATECHOLAMINE METABOLISM IN A FUNCTIONAL NEURAL TUMOR *

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In recent years the association of chronic diarrhea with tumors of neural origin has been reported by several authors (1–5). Green, Cooke and Lattanzi (5), in their report of three such cases, focused attention on the possible secretory nature of these tumors by emphasizing the cessation of symptomatology that results following their operative removal. Evidence of increased production of catecholamines in such cases was then presented simultaneously by Dicke and coworkers (6) and by Greenberg and Gardner (7). More recently, two additional cases with similar results have been reported (8, 9).

As noted by Sjoerdsma, the study of catecholamine metabolism in humans can, perhaps, be most easily accomplished by the investigation of the biosynthesis and fate of catecholamines in patients with pheochromocytoma (10). Urine from such patients has proved to be a good source for the identification of the metabolites of pressor amines.

The demonstration of the hormonal nature of other tumors of nervous tissue opens up a new area for the study of catecholamine metabolism by human nervous tissue. This paper is a report of studies carried out in a two and one-half year old girl who manifested chronic, severe diarrhea and a normal postoperative course in association with a posterior mediastinal ganglioneuroma.

CASE HISTORY

C.M., SMH 423041-3, a 2.5 year old white female, was admitted to Syracuse Memorial Hospital on May 24, 1959, with a chief complaint of chronic diarrhea of 1 year's duration. At the age of 15 months she developed a watery diarrhea, 6 to 8 stools per day, that persisted up to the time of admission, associated with increasing abdominal distention and failure to gain weight for the preceding 8 months. There was no anorexia; an intake of 8 to 10 glasses of water per day was noticed with an apparent polyuria. There was no history of increased perspiration, skin rashes, flushing or episodic abdominal pain. Coldness of the distal parts of the extremities was not observed, and she remained afebrile throughout. Prenatal and birth histories were entirely normal, as were her early development and feeding. There are 4 normal siblings in the family and a negative family history for significant abnormalities or diseases.

Physical examination. Observation revealed a small, alert wiry girl with a protuberant abdomen and a paucity of subcutaneous adipose tissue. Blood pressure was 105/70; weight 21 pounds; height 32.5 inches; head circumference 18.25 inches; head to pubis 18.75 inches; arm span 31.38 inches; upper/lower ratio, 1.35. Significant findings were limited to the abdomen which was distended and tympanitic and quiet to auscultation. No visceromegaly was present; rectal examination was normal; the stool was watery, brown and nonfatty.

Radiological studies. Radiographic and fluoroscopic examination of the chest with barium swallow revealed a large circumscribed area of increased density in the posterior superior mediastinum. An extrapleural shadow was present in both apices (Dr. Edward Carsky). Examination of both hands and wrists showed normal bone architecture and a skeletal maturation corresponding to the chronological age.

Serum chemistries. Chemical measurements on serum are recorded in Table I. Hypokalemia was present. Associated with this was a failure to conserve water; i.e., after a 12 hour period of oral fluid deprivation, the urine specific gravity was 1.009. Urine collections for 6 consecutive preoperative 24-hour periods revealed volumes of 960, 765, 740, 550, 1005 and 960 ml, with specific gravities ranging between 1.005 and 1.010. Hypophosphatemia of marked degree was found, in association with slightly diminished alkaline phosphatase activity.

Hospital course. The patient continued to manifest persistent watery diarrhea until surgery. She showed no hypertension throughout her hospital stay. On June 5, 1959, a right thoracotomy was performed by Dr. Lawrence K. Pickett with the removal of a smooth, encapsulated tumor mass from the right superior mediastinum, measuring 4.5 × 1.5 × 2 cm and weighing 42 g. Part of the

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† Fellow of the National Foundation; portions of these studies were carried out under the fellowship at the Fysiologiska Institutionen, Karolinska Institutet, Stockholm, Sweden. Present address: Department of Pediatrics, Stanford University School of Medicine, Palo Alto, Calif.

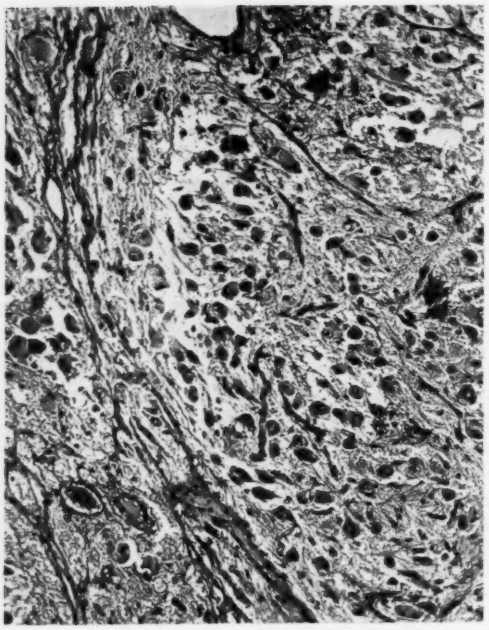


FIG. 1. PHOTOMICROGRAPH OF GANGLIONEUROMA FROM PATIENT C.M. (× 120). Note numerous well differentiated ganglion cells.

tumor was frozen in dry ice-alcohol within 15 minutes after removal. The histologic appearance of the tumor was characteristic of a ganglioneuroma with focal calcification (Dr. John T. Prior), as shown in Figure 1. The postoperative course was completely uneventful with immediate cessation of diarrhea and resumption of a normal stool pattern.

METHODS

1. Assay of urinary hormones and metabolites. The major metabolite of 5-hydroxytryptamine (serotonin), 5-hydroxyindoleacetic acid (5-HIAA), was determined by the method of Udenfriend, Titus and Weissbach (11). Free norepinephrine and epinephrine were determined on the same specimens (collected in 5 ml 6 N HCl and maintained in a frozen state) by two means: *a*) the method of von Euler and Floding, using iodine as the oxidant (12) and *b*) the method of von Euler and Lishajko (13).

Dopamine was determined according to Carlsson and Waldeck (14), after elution from an alumina column with 0.25 N acetic acid. Norepinephrine, epinephrine, dihydroxyphenylacetic acid (dopac) and metanephrine produced less than 2 per cent fluorescence in equimolar amounts when compared with dopamine under the conditions of this method.

3-Methoxy-4-hydroxymandelic acid (VMA), a major metabolite of norepinephrine (and epinephrine) was determined by the semiquantitative paper chromatographic method of Armstrong, Shaw and Wall (15, 16). Confirmatory determinations were done by the method of von Studnitz and Hanson (17).[1] Homovanillic acid, a major metabolite of both dopa and dopamine, was determined by high voltage paper electrophoresis (18).

3-Methoxy-4-hydroxyphenylalanine (3-methoxydopa), a newly identified amino acid, was isolated in the preoperative urine,[1] confirming a previous finding in a similar case (18).

2. Biochemical studies of the tumor., Five per cent trichloroacetic acid extracts of the tumor were passed through an alumina column at pH 8.3 and eluted with 0.25 N acetic acid. Norepinephrine, epinephrine and dopamine were then determined by previously described methods (13, 14).

Approximately 3 g of the tumor was extracted and passed through an alumina column as above; the eluate was evaporated *in vacuo* at less than 35° C to 2 ml, extracted twice with 5 vol of butanol. After evaporation, the butanol extract was applied to Whatman no. 1 chromatographic paper. After a 24 hour run, descending,

[1] These determinations were kindly done by Dr. Wilfried von Studnitz, Department of Clinical Chemistry, University of Lund, General Hospital, Malmö, Sweden.

TABLE I

Serum chemistries demonstrating hypokalemia and hypophosphatemia

Date	Na^+	K^+	CO_2	Cl^-	BUN	Total proteins	Serum albumin	Ca	P	Alk. phosphatase
	mEq/L	*mEq/L*	*mEq/L*	*mEq/L*	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*	*B-L units**
5/26/59	140	2.9	16	106	30					
5/27/59					23					
5/29/59	135	3.0	22	99				10.6	1.6	
6/1/59†	128	3.9	18	90						2.4
6/3/59†	139	3.3	18.5	98		7.2	5.8		2.8	2.6
6/5/59	Surgery									
6/10/59	133	5.6	26	97	14	6.8	4.5	10.6	4.2	

* The method of Bessey, Lowry and Brock was used for serum alkaline phosphatase (J. biol. Chem. 1946, **164**, 321). The normal range for children in this laboratory is 2.8 to 6.7 B-L units.

† During the period 6/1 to 6/3, oral K^+ supplement was 45 mEq/day.

TABLE II

Summary of determinations of catecholamines and metabolites in pre- and postoperative urine (see text)

	Norepinephrine		Epinephrine		3-Methoxy-4-hydroxy-mandelic acid			Dopamine	Homovanillic acid	
	A	B	A	B	C		D			
	μg/24 hrs		*μg/24 hrs*		*μg/mg urinary creatinine*		*mg/24 hrs*	*μg/24 hrs*	*μg/mg urinary creatinine*	*mg/24 hrs*
Preoperative	148	232	3.6	0.0	25	90	13.0	2,833	402	58
Postoperative	17.1	22	0.65	2.9	1.25	14.5	1.4	100	120	12.5
Normal values	8.0 ± 4.0		1.2 ± 0.5		1.5–3.0*	2.8–7.5*		5–10X norepinephrine values†	Not available	

* These values refer to adults and children above 3 years of age. Below that age the values are slightly higher; creatinine is used as a basis for comparison, with the relatively lower muscle mass of young children resulting in an apparently increased excretion of 3-methoxy-4-hydroxymandelic acid.

† Normal values obtained by use of same method in newborns (25) and adults (26).

with butanol saturated with 1 N HCl as the mobile phase, the chromatogram was sprayed with ethylenediamine 10 per cent (vol/vol); the reference spots, representing approximately 10 μg pure substance, were located by exposure to ultraviolet light and the paper was cut into 2 × 1 cm strips, eluted overnight in dilute NH_4OH (pH 10), and the fluorescence was read in a Coleman spectrofluorometer.

The histamine content of the tumor was determined [2] by the fluorescent method of Shore, Burkhalter and Cohn (19).

3. In vitro biologic activity of the tumor. A trichloroacetic acid extract of 0.192 g of the tumor was washed 3 times with peroxide-free ether and taken to pH 4.5 with 1 N NaOH, then bioassayed on a hen's isolated rectal cecum preparation, according to the method of von Euler (20).

4. Assay of catechol-O-methyl transferase activity. When the soluble supernatant fraction from a wide variety of tissues is incubated with S-adenosylmethionine and epinephrine, in the presence of a cation (especially Mg^+), metanephrine (3-methoxy derivative of epinephrine) is formed, as described by Axelrod (21–23). Tissue, representing 0.5778 g wet weight of tumor, was assayed for its ability to form metanephrine according to the method of Axelrod and Tomchick (22). Attempts were made to isolate normetanephrine from the tumor by paper chromatography of organic solvent extracts of the filtrate of trichloroacetic extracts of the tumor, which had been passed through alumina columns. Extractions of the filtrates were done with butanol, followed by heptane, and also with isoamylalcohol: toluene, 3: 2, both at pH 10, with re-extraction in both cases with a small volume of 0.05 N HCl.

[2] These determinations were kindly performed by Dr. Helen Treadway Graham, Department of Pharmacology, Washington University School of Medicine, St. Louis, Mo.

RESULTS

In three consecutive preoperative urine collections, 1.61, 1.69 and 2.06 mg of 5-HIAA per 24 hours were present. Normal levels for children of comparable age are not available; however, normal adult levels, using the same method, range from 1.7 to 5.5 mg per 24 hours (24).

The preoperative excretion of norepinephrine, VMA, dopamine and homovanillic acid was elevated when compared with normal values and/or postoperative levels. The results of these determinations are summarized in Table II. Columns designated A and B refer, respectively, to figures derived from the methods of von Euler and Floding (12) and von Euler and Lishajko (13). The normal levels, obtained by the use of the former method, are abstracted from Zeisel and Kuschke (27) and are in agreement with our unpublished observations. The slightly elevated postoperative levels are believed to be of no significance in view of the normal course of the patient during the six months since surgery.

The identity of the activation and fluorescent spectra (as determined in an Aminco-Bowman spectrophotofluorometer) of dopamine in the preoperative urine with standard dopamine is demonstrated in Figure 2.

The marked elevation in the urinary excretion of VMA was found when determined by both methods used; in Table II, C refers to the method of Armstrong (16) and D to the method of von Studnitz and Hanson (17). The discrepancy in

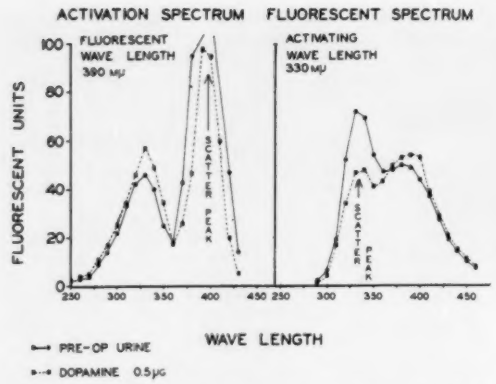


FIG. 2. ACTIVATION AND FLUORESCENT SPECTRA OF AN ALIQUOT OF PREOPERATIVE URINE, DEMONSTRATING THE SIMILARITY TO STANDARD DOPAMINE. See text for methods.

amounts found by the two methods is probably based on a difference in methodology. Homovanillic acid was elevated when compared with the postoperative value; no normal values have been established.

The tumor itself contained 8.6 μg norepinephrine, 0.074 μg epinephrine and 2.09 dopamine per g wet weight. Figure 3 depicts the identity of fluorescent peaks of an extract of the tumor with standard norepinephrine and dopamine, when chromatographed in butanol: HCl (see Discussion). Two separate homogenates of the tumor were assayed for histamine content, the second of which contained more connective tissue than the

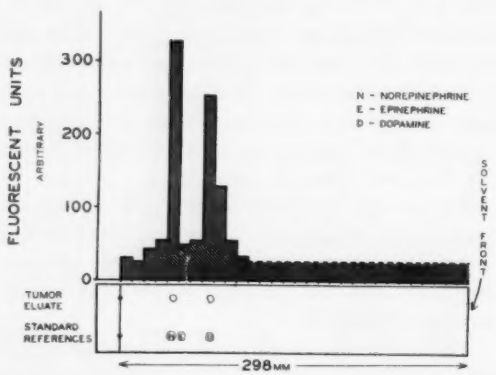
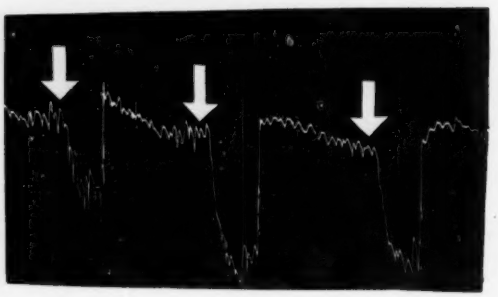


FIG. 3. QUANTITATIVE PAPER CHROMATOGRAPHY OF AN ELUATE FROM THE GANGLIONEUROMA. Ethylenediamine condensation method; N = norepinephrine, E = epinephrine, D = dopamine.



0.1 cc Tumor Extract	0.2 cc Tumor Extract	200 mμg NE

(0.1 cc extract = 6.3 mg tumor wet wt.)

FIG. 4. BIOASSAY OF TUMOR EXTRACT ON HEN'S ISOLATED RECTAL CECUM, METHOD OF VON EULER (20).

first. Respectively, the values found were 0.37 and 0.261 μg per g wet weight of tissue. These values correspond to the content found in normal brain tissue by the same method.

When a crude extract of the tumor was bioassayed on a hen's isolated rectal cecum, relaxation of the intestinal smooth muscle was produced, as shown in Figure 4. By this method, 15.2 μg of norepinephrine-equivalents per g wet weight of tumor was calculated to be present.

As a means of studying possible mechanisms governing catecholamine release as a result of this tumor, saline extracts were prepared of two 100 mg wet weight portions of the tumor and injected into male rats. No increase in catecholamine excretion was found, suggesting, at least according to this procedure, the absence of any substance stimulating catecholamine excretion from sites other than the tumor itself.

TABLE III

In vitro assay for cathechol-O-methyl transferase activity *

	Metanephrine formed
	μg/50 mg tissue/hr
Tumor	0.0
Control (rat liver)	3.29
S-adenosylmethionine omitted	0.0

* Supernatant from tumor and control (rat liver) incubated with L-epinephrine (0.3 μmole), S-adenosylmethionine (0.2 μmole) and $MgCl_2$ (10 μmoles), with phosphate buffer at pH 7.8 (50 μmoles) at 38° C for 1 hour. Total volume, 1 ml.

Table III indicates the absence of catechol-O-methyl transferase activity in the tumor. It should be noted that the activity of the control rat liver was somewhat lower than that reported in the studies of Axelrod and Tomchick (21, 22). Thus, some enzyme activity of the tumor might not have been detected. However, no normetanephrine could be isolated from the tumor by paper chromatographic techniques. It appears, then, that the tumor was unable to metabolize the norepinephrine formed within it, at least by the important and perhaps primary mechanism of O-methylation (23).

DISCUSSION

Increased amounts of norepinephrine and epinephrine excretion have been found in cases of neuroblastoma by Isaacs, Medalie and Politzer (28) and Mason and associates (29). The presence of pressor amines in the tissue of a neuroblastoma was reported recently by Graham (30). Increased excretion of VMA in cases of ganglioneuroma associated with chronic diarrhea has now been found on four occasions (6–9). These data have been amplified, in this report, by the finding of increased urinary excretion of dopamine and homovanillic acid and the presence of norepinephrine and dopamine in the tumor. Cells comprising the sympathetic nervous system and chromaffin tissue, both in the adrenal medulla and extramedullary in location, are derived from a similar embryologic origin (31). It is now apparent that tumors arising from such cells, whether benign or malignant, can form catecholamines and act as true functional or hormone-producing neoplasms.

The original hypothesis of Blaschko (32) on the metabolic route for the biosynthesis of catecholamines is shown with heavy arrows in Figure 5. This has been supported by most studies during the past decade [see reviews by Blaschko (33) and Kirshner (34)]. Of particular interest with respect to this report are the studies of Goodall and Kirshner (35) who demonstrated the formation of norepinephrine, but only very little epinephrine, from tyrosine in sympathetic nerves and ganglia. That norepinephrine can be formed from dopa and dopamine in pheochromocytoma tissue has also been shown by Sjoerdsma, Leeper, Terry and Udenfriend (36) and Gélinas, Pellerin and D'Iorio (37). The findings, in the present report, of dopamine and norepinephrine in the tumor tissue, increased urinary excretion of homovanillic acid [a major metabolite of both dopa and dopamine (23)], and the presence of the O-methylated derivative of dopa in the preopera-

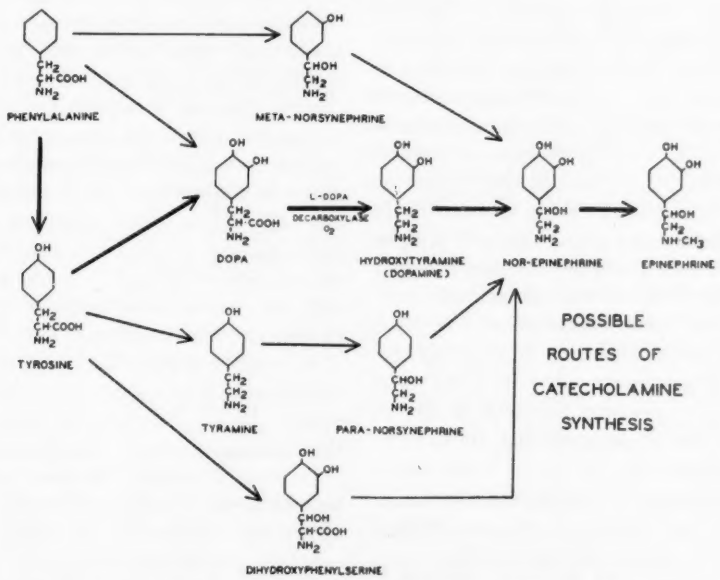


FIG. 5. POSSIBLE ROUTES OF CATECHOLAMINE BIOSYNTHESIS.

tive urine provide indirect evidence of the existence of the dopa → dopamine → norepinephrine pathway in this ganglioneuroma. Attention should be directed to the report of Senoh, Creveling, Udenfriend and Witkop (38) which describes the (auto)oxidation of dopamine to form 2,4,5-trihydroxyphenethylamine, a substance that cannot be separated from norepinephrine by chromatography or recrystallization except after treatment with methanolic hydrogen chloride. The relative specificity of the oxidation with ferricyanide substantiates the existence of norepinephrine in the tumor studied in this report.

Recent studies have suggested that dopamine may have a function other than as an intermediate in the synthesis of norepinephrine and epinephrine. Indeed, dopamine has been recognized as a normal constituent in human urine for many years (39, 40). In the brain of all mammalian species studied, norepinephrine and dopamine are present in approximately equal quantities (41). There is, however, a disproportionate distribution of catecholamines in the central nervous system; dopamine is present in high concentration in the caudate nucleus, whereas norepinephrine is most abundant in the hypothalamus. Dopamine, similarly, has been demonstrated in adrenergic nerves (42) and has been isolated in tumor tissue (pheochromocytomas) previously (43–45). The occurrence in ruminants of large amounts of dopamine in lung, spleen (46), duodenum and liver is of interest in view of the close correlation between the distribution of dopamine and a special type of chromaffin cell (47). Such cytological correlations have not, however, been found in either brain or sympathetic nervous tissue. While it seems likely that dopamine has its own physiologic role in those tissues in which its concentration is much higher than its "successor" in the metabolic pathway, norepinephrine, there is no evidence as yet that dopamine serves any physiologic role in the peripheral sympathetic nervous system other than precursor to norepinephrine. Whether this may be altered in the case of dopamine-producing tumors remains to be determined.

Is it possible to relate, in a cause and effect manner, the biochemical findings and symptomatology in the case? The abolition of both symptoms and elevated excretion of catecholamines and metabolites after removal of the tumor is suggestive of a relationship between the two phenomena. Indeed, the precise mechanism of diarrhea in such cases awaits clarification. Increased excretion of catecholamines, as in cases of pheochromocytoma, has often been reported in the absence of disturbed bowel function. The hypokalemia observed in this patient may be secondary to chronic diarrhea or may be associated with the prolonged elevation of blood organic acids. The relationships between catecholamines and potassium balance as reviewed by Ellis (48), are too unclear to support generalizations.

The evidence obtained in this study suggests that the tumor was unable to form the O-methylated metabolites of catecholamines. If so, the O-methylated metabolites found in excess in the preoperative urine must have been formed elsewhere in the patient. The enzyme, catechol-O-methyl transferase, is widely distributed throughout the body, having its highest activity in the liver (22).

SUMMARY

The syndrome of chronic diarrhea in the presence of tumors of neural origin has been found to be associated with increased production of catecholamines and their metabolites. In this report, increased urinary excretions of norepinephrine, 3-methoxy-4-hydroxymandelic acid, dopamine and homovanillic acid were found preoperatively in a 2.5 year old girl who exhibited chronic diarrhea in association with a posterior mediastinal ganglioneuroma. All values returned to near normal following operative removal of the tumor in conjunction with a cessation of symptomatology. The tumor contained norepinephrine and dopamine, as shown by fluorescent and chromatographic techniques. Evidence was obtained that the tumor was incapable of forming the O-methylated metabolites of catecholamines.

Although it appears likely that the production of catecholamines by the tumor is related to the symptom of chronic diarrhea, the exact mechanism of this relationship awaits clarification.

In cases of chronic diarrhea and/or suspected tumors of neural origin, the determination of norepinephrine, dopamine and 3-methoxy-4-hydroxymandelic acid in the urine should prove to be of considerable aid in diagnosis and management.

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REFERENCES

1. Lanckneus, J. Over een neurogens thoraxtumor. Maandschr. Kindergeneesk. 1954, **22**, 145.
2. Bigler, J. A., and Hoyne, A. Ganglioneuroma. Report of 2 cases with a review of the literature. Amer. J. Dis. Child. 1932, **43**, 1552.
3. Hawfield, H. H., and Daisley, G. W., Jr. Report of case of a functional adrenal ganglioneuroma (case report number 231). Clin. Proc. Child. Hosp. (Wash.) 1952, **8**, 98.
4. Uhlmann, E. M., and von Essen, C. Neuroblastoma (neuroblastoma sympatheticum). Pediatrics 1955, **15**, 402.
5. Green, M., Cooke, R. E., and Lattanzi, W. Occurrence of chronic diarrhea in 3 patients with ganglioneuroma. Pediatrics 1959, **23**, 951.
6. Dicke, W. K., Kingma, B. E., Wadman, S. K., de Jongh, S. E., and Noach, E. L. Ganglioneuroma as a cause of chronic diarrhea. Presented at the IXth Internat. Congr. of Paediatrics, Montreal, July, 1959.
7. Greenberg, R. E., and Gardner, L. I. New diagnostic test for neural tumors of infancy: Increased urinary excretion of 3-methoxy-4-hydroxymandelic acid and norepinephrine in ganglioneuroma with chronic diarrhea. Pediatrics 1959, **24**, 683.
8. Stickler, G. B., Hallenbeck, G. A., and Flock, E. V. Ganglioneuroblastoma associated with chronic diarrhea and increased excretion of catecholamines: Preliminary report. Proc. Mayo Clin. 1959, **34**, 548.
9. Voorhess, M. L., and Gardner, L. I. Studies of catecholamine metabolism in neural tumors (abstract). A.M.A. Amer. J. Dis. Child. 1960. In press.
10. Sjoerdsma, A. Catecholamine metabolism in patients with pheochromocytoma. Pharmacol. Rev. 1959, **11**, 374.
11. Udenfriend, S., Titus, E., and Weissbach, H. The identification of 5-hydroxy-3-indoleacetic acid in normal urine and a method for its assay. J. biol. Chem. 1955, **216**, 499.
12. von Euler, U. S., and Floding, I. Diagnosis of pheochromocytoma by fluorimetric estimation of adrenaline and noradrenaline in urine. Scand. J. clin. Lab. Invest. 1956, **8**, 288.
13. von Euler, U. S., and Lishajko, F. The estimation of catechol amines in urine. Acta physiol. scand. 1959, **45**, 122.
14. Carlsson, A., and Waldeck, B. A fluorimetric method for the determination of dopamine (3-hydroxytyramine). Acta physiol. scand. 1958, **44**, 293.
15. Armstrong, M. D., Shaw, K. N. F., and Wall, P. E. The phenolic acids of human urine; paper chromatography of phenolic acids. J. biol. Chem. 1956, **218**, 293.
16. Armstrong, M. D. Estimation of 3-methoxy-4-hydroxymandelic acid for the detection of pheochromocytoma. Privately circulated by the author, Fels Research Institute, Yellow Springs, Ohio, 8 pp.
17. von Studnitz, W., and Hanson, A. Determination of 3-methoxy-4-hydroxymandelic acid in urine by high-voltage paper electrophoresis. Scand. J. clin. Lab. Invest. 1959, **11**, 101.
18. von Studnitz, W., and Vendsalu, A. Unpublished data.
19. Shore, P. A., Burkhalter, A., and Cohn, V. H. Fluorimetric estimation of tissue histamine. Fed. Proc. 1959, **18**, 444.
20. von Euler, U. S. Noradrenaline; Chemistry, Physiology, Pharmacology, and Clinical Aspects. Springfield, Ill., Charles C Thomas, 1956.
21. Axelrod, J. O-Methylation of epinephrine and other catechols *in vitro* and *in vivo*. Science 1957, **126**, 400.
22. Axelrod, J., and Tomchick, R. Enzymatic O-methylation of epinephrine and other catechols. J. biol. Chem. 1958, **233**, 702.
23. Axelrod, J. Metabolism of epinephrine and other sympathomimetic amines. Physiol. Rev. 1959, **39**, 751.
24. Kowlessar, O. D., Williams, R. C., Law, D. H., and Sleisenger, M. H. Urinary excretion of 5-hydroxyindolacetic acid in diarrheal states with special reference to nontropical sprue. New Engl. J. Med. 1958, **259**, 340.
25. Greenberg, R. E. Unpublished observations.
26. Drujan, B. D., Sourkes, T. L., Layne, D. S., and Murphy, G. F. The differential determination of catecholamines in urine. Canad. J. Biochem. 1959, **37**, 1153.
27. Zeisel, H., and Kuschke, H. J. Die Catechinamine Adrenalin und Noradrenalin im Harn des Kindes. Klin. Wschr. 1959, **37**, 1168.
28. Isaacs, H., Medalie, M., and Politzer, W. M. Noradrenaline-secreting neuroblastoma. Brit. med. J. 1959, **1**, 401.
29. Mason, G. A., Hart-Mercer, J., Millar, E. J., Strang, L. B., and Wynne, N. A. Adrenaline-secreting neuroblastoma in an infant. Lancet 1957, **2**, 322.
30. Graham, J. D. P. Pressor amines and neuroblastoma. Nature (Lond.) 1959, **183**, 1733.
31. Kuntz, A. The Autonomic Nervous System, 4th ed. Philadelphia, Lea & Febiger, 1953.
32. Blaschko, H. The specific action of L-dopa decarboxylase. J. Physiol. (Lond.) 1939, **96**, 50P

33. Blaschko, H. The development of current concepts of catecholamine formation. Pharmacol. Rev. 1959, **11**, 307.
34. Kirshner, N. Biosynthesis of adrenaline and noradrenaline. Pharmacol. Rev. 1959, **11**, 350.
35. Goodall, M., and Kirshner, N. Biosynthesis of epinephrine and norepinephrine by sympathetic nerves and ganglia. Circulation 1958, **17**, 366.
36. Sjoerdsma, A., Leeper, L. C., Terry, L. L., and Udenfriend, S. Studies on the biogenesis and metabolism of norepinephrine in patients with pheochromocytoma. J. clin. Invest. 1959, **38**, 31.
37. Gélinas, R., Pellerin, J., and D'Iorio, A. Biochemical observations of a chromaffine tumour. Rev. canad. Biol. 1957, **16**, 445.
38. Senoh, S., Creveling, C. R., Udenfriend, S., and Witkop, B. Chemical, enzymatic and metabolic studies on the mechanism of oxidation of dopamine. J. Amer. chem. Soc. 1959, **81**, 6236.
39. Holtz, P., Credner, K., and Koepp, W. Die enzymatische Entstehung von Oxytyramin im Organismus und die physiologische Bedeutung der Dopadecarboxylase. Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak. 1942, **200**, 356.
40. von Euler, U. S., Hamberg, U., and Hellner, S. β-(3:4-Dihydroxyphenyl) ethylamine (hydroxytyramine) in normal human urine. Biochem. J. 1951, **49**, 655.
41. Carlsson, A. The occurrence, distribution and physiological role of catecholamines in the nervous system. Pharmacol. Rev. 1959, **11**, 490.
42. Schümann, H. J. Nachweis von Oxytyramin (Dopamin) in sympathischen Nerven und Ganglien. Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak. 1955, **227**, 566.
43. Weil-Malherbe, H. Phaeochromocytoma; catechols in urine and tumor tissue. Lancet 1956, **2**, 282.
44. McMillan, M. Identification of hydroxytyramine in a chromaffin tumor. Lancet 1956, **2**, 284.
45. Manger, W. M., Flock, E. V., Berkson, J., Bollman, J. L., Roth, G. M., Baldes, E. J., and Jacobs, M. Chemical quantitation of epinephrine and norepinephrine in thirteen patients with pheochromocytoma. Circulation 1954, **10**, 641.
46. von Euler, U. S., and Lishajko, F. Dopamine in mammalian lung and spleen. Acta physiol. pharmacol. neerl. 1957, **6**, 295.
47. Bertler, Å., Falck, B., Hillarp, N. Å., Rosengren, E., and Torp, A. Dopamine and chromaffin cells. Acta physiol. scand. 1959, **47**, 251.
48. Ellis, S. Relation of biochemical effects of epinephrine to its muscular effects. Pharmacol. Rev. 1959, **11**, 469.

RESPIRATORY MECHANICS DURING FORWARD ACCELERATION *

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Tolerance to headward and footward accelerations is seriously limited by cardiovascular impairment. During forward acceleration, when the subject is so oriented that the inertial effect experienced is transverse to the long axis of the body and directed front to back, toleration is increased, but substernal pain, sensation of pressure on the chest and abdomen, dyspnea and difficulty in inspiring (1) become the limiting factors. Vital capacity and maximal breathing capacity decrease while respiratory frequency and minute volume increase (2). X-rays of the thorax reveal elevation of the diaphragm and shortening of the anteroposterior diameter resembling forced expiration (13). These changes in pulmonary physiology are similar to those that have been described during negative pressure breathing (4–7). Since the net inertial effect of forward acceleration resembles a compressive force applied to the body while the subject continues to breath ambient air, it seemed reasonable to assume that forward acceleration simulates negative pressure breathing in a manner analogous to breathing ambient air while submerged under water (5, 8). In order to test this hypothesis, measurements were made of the pressure-volume relationships and the changes in lung volumes occurring during forward acceleration.

METHODS

1. Subjects. Four healthy adult males between the ages of 23 and 30, experienced in riding the free-swinging cab of the human centrifuge at the Biomedical Laboratory, were used as subjects. Measurements were obtained while the subjects were lying on a nylon net seat facing the center of rotation with the trunk perpendicular to the inertial force, hips and knees flexed 90° and head and back tilted forward 12° from the horizontal (Figure 1).

2. Lung volumes. Vital capacity, expiratory reserve, inspiratory reserve and tidal air were measured at 1, 2, 3 and 4 G from a 9 L Benedict Roth recording spirometer with the CO_2 absorber in place. Residual volume at 1 and 4 G was determined by the method of Lundsgaard and Van Slyke (9) as modified by Rahn, Fenn and Otis (10). Residual volume at 4 G was measured by having the subject expire to his maximal end-expiratory position while at 4 G. The centrifuge was then rapidly stopped while the subject maintained this lung volume. The equilibration with 100 per cent oxygen required by the method was then carried out at 1 G. The concentration of N_2 in the expired gas sample was determined by a Waters Conley nitrogen analyzer.

The relative change in the residual air, but not its absolute volume, was measured in the following manner. At 1 G the subjects expired into a spirometer to their maximal end-expiratory position. While still in communication with the spirometer, they were then rapidly accelerated to 4 G. Any shift in the spirometer tracing then represented a change in residual volume.

3. Static relaxation pressure-volume curves. Lung volumes were measured as above and tabulated as percentage of vital capacity. To measure intrapulmonic pressure a polyethylene tube 0.5 cm in diameter and 40 cm in length was fitted into one nostril through a sponge rubber stopper. The tube was attached to a Statham strain gage pressure transducer and the results transcribed on a Sanborn multichannel recorder. Control values at rest, or 1 G, were obtained by the subject's inspiring from the maximal end-expiratory position to a given lung volume. Then while the subject held his breath at the desired lung volume, the mouthpiece was removed, and with mouth closed, glottis open and subject relaxed, intrapulmonic pressure was recorded. Each subject furnished approximately 130 pressure readings over the entire range of his vital capacity.

The above procedure was repeated in order to obtain the relaxation pressure-volume curves during forward acceleration. After a lung volume was obtained, the mouthpiece removed and the subject relaxed, the centrifuge was accelerated to 2, 3 and 4 G plateaus for 2 to 3 seconds each. Intrapulmonic pressure was then recorded for each lung volume at each G level.

In order to verify the pressure measurements obtained at each acceleration plateau, a few measurements of intrapulmonic pressure were obtained by first accelerating to the desired level and then obtaining a lung volume and recording a pressure. No essential difference was noted between the two methods. However, because of the

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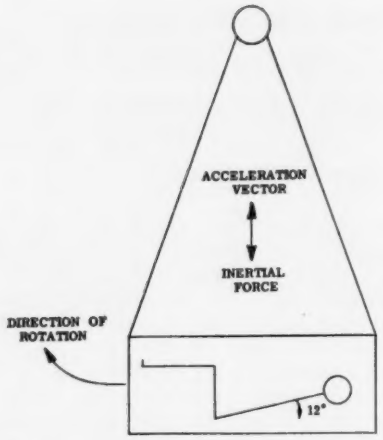

FIG. 1. ORIENTATION OF THE SUBJECT IN THE HUMAN CENTRIFUGE DURING FORWARD ACCELERATION WITH A 12° BACK ANGLE FROM THE HORIZONTAL.

difficulty in obtaining the curves in this manner, this method was not used throughout. Also, since the subjects could obtain larger lung volumes at 1 G than they could while being accelerated, the first method enabled us to extend the relaxation pressure-volume curves to 85 per cent of the 1 G vital capacity at all accelerations.

4. Dynamic pressure-volume loops. Estimation of the total pulmonary work of breathing at 1 and 4 G was obtained by measuring the areas of pressure-volume respiratory loops (11). Each subject was instructed to relax while being ventilated by a Seeler paramedic resuscitator (type XMA-4), whose line pressure and inflow resistance remained the same during both levels of acceleration. Mask pressure ranged between 0 and 30 mm of Hg at 1 and 4 G. Changes in volume were measured by electrical integration of the signal from a pneumotachygraph linear for flow rates between 0 and 107 L per minute. Pressure was measured from a tight-fitting face mask by polyethylene tubing connected to a Statham pressure transducer. The pressure-volume respiratory loops were recorded directly on an Electronics for Medicine recorder from the integrated flow signal and the mask pressure transducer signal. The recorder and the pneumotachygraph were calibrated dynamically with a constant-volume pump. The response was constant and independent of frequency. Pressure-volume respiratory loops were then recorded at both 1 and 4 G for all subjects.

The areas measured to determine the work of breathing at 1 and 4 G are shown in Figure 2. The total work was divided into its elastic and non-elastic components, and calculated by the usual method (5, 11, 12). For example, at 1 G elastic work is represented by the area ACD and the non-elastic work by the cross-hatched area. At 4 G, since the pressure-volume curve as shown in Figure 4 is displaced 15 mm of Hg to the right by the compressive force of acceleration, additional work must be done to overcome this force. Therefore, the origin of the pressure-volume loop at 4 G was placed at a positive 15 mm of Hg to illustrate this additional work of breathing. However, the recorded mask pressure at the beginning of inspiration regardless of the acceleration was actually zero. This is comparable with the method used by Rahn, Otis, Chadwick and Fenn (5) to calculate the work of respiration during positive pressure breathing. Elastic work is, therefore, calculated to be equal to the area EACD, while non-elastic work remains the cross-hatched area. Although, for simplicity, the work necessary to overcome this compressive force is included with the total

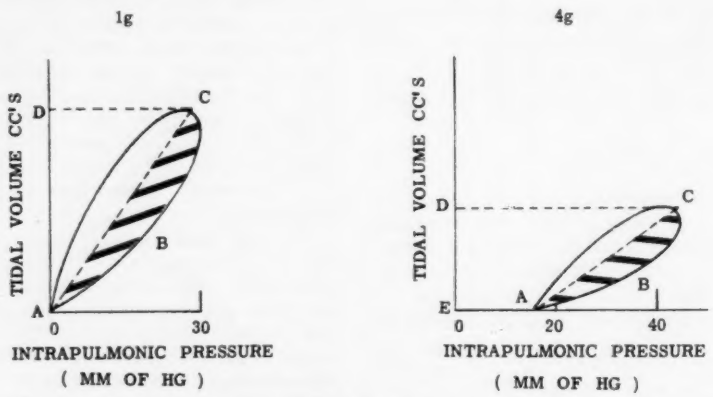

FIG. 2. SCHEMATIC PRESSURE-VOLUME RESPIRATORY DIAGRAMS TO ILLUSTRATE THE AREAS MEASURED FOR CALCULATION OF THE TOTAL WORK OF BREATHING (LUNG AND CHEST BELLOWS) DURING CONTROL (1 G) AND ACCELERATION (4 G). The loop at 4 G has been displaced 15 mm of Hg to the right to represent the shift of the pressure-volume curve by acceleration. Elastic work represents the area ACD at 1 G and EACD at 4 G, while the cross-hatched areas represent non-elastic work.

TABLE I

Changes in lung volumes during acceleration *

Acceleration	T.L.C.		V.C.		E.R.		I.R.		Tidal		R.V.	
G	*cc*	%†	*cc*	%	*cc*	%	*cc*	%	*cc*	%	*cc*	%
1	5,004		3,875 ± 343		761 ± 135		2,333 ± 191		781 ± 107		1,129 ± 143	
2			3,488 ± 437	90	502 ± 128	66	2,468 ± 183	106	518 ± 108	66		
3			3,219 ± 430	83	419 ± 123	55	2,282 ± 258	98	518 ± 108	66		
4	3,932	79	2,629 ± 460	68	360 ± 94	47	1,785 ± 310	77	484 ± 71	62	1,303 ± 165	116

* Mean of four subjects with SE. Abbreviations used: T.L.C. = total lung capacity; V.C. = vital capacity; E.R. = expiratory reserve; I.R. = inspiratory reserve; R.V. = residual volume.
† Per cent of 1 G value.

elastic work, it must be kept in mind that this work may not be elastic in its true meaning.

From these pressure-volume loops, total dynamic pulmonary compliance, i.e., the distensibility of the entire respiratory apparatus, including lungs and chest bellows, was measured from the slope of the line connecting points of zero air flow.

RESULTS

1. Lung volumes. The changes in lung volumes occurring during acceleration are shown in Table I and Figure 3 along with the standard errors and the percentages of the 1 G value. All the lung volumes decrease more or less proportionately (with the exception of residual volume which shows no significant change) by either of the two methods used.

2. Static relaxation pressure-volume relationships. The weighted mean static relaxation pressure-volume curves for 1, 2, 3 and 4 G are plotted in Figure 4, with the volumes expressed as percentage of the 1 G vital capacity. As acceleration increases, intrapulmonic pressure increases for a constant volume displacing the relaxation pressure-volume curve progressively downward and to the right. The difference between each curve by the paired *t* test is significant with a $p < 0.01$ (13).

The slopes of the relaxation pressure-volume curves for each G appear identical. However, if static compliance is measured from the end-expiratory position at each G and over a pressure change of 5 mm of Hg, the static total pulmonary compliance becomes progressively smaller as ac-

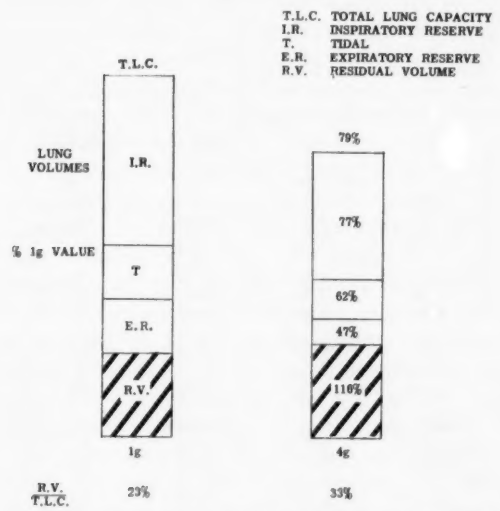

FIG. 3. SCHEMATIC REPRESENTATION OF THE CHANGES OCCURRING IN TOTAL LUNG CAPACITY AND ITS SUBDIVISIONS FROM CONTROL (1 G) TO 4 G FORWARD ACCELERATION.

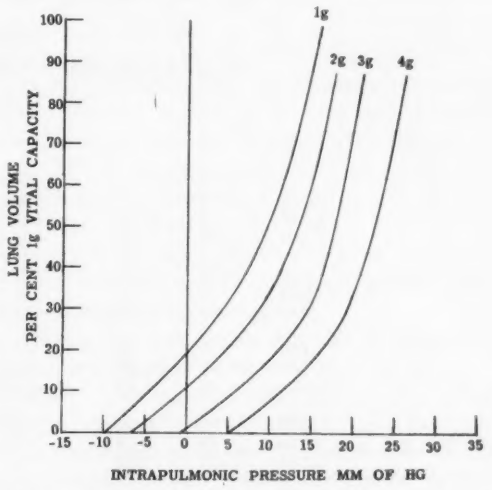

FIG. 4. THE STATIC RELAXATION PRESSURE-VOLUME CURVES DURING CONTROL (1 G), AND 2, 3 AND 4 G FORWARD ACCELERATION. All lung volumes were obtained at 1 G (see Methods).

TABLE II

*The work of breathing and total dynamic compliance * at 1 G*

Subject	Tidal	Pt	f	Ve	Work of breathing			Dynamic compliance
					Elastic	Non-elastic	Total	
	cc	*cm H₂O*		*L/min*		*kg/m/min*		*cc/cm H₂O*
J.F.	2,220	27	7	15.5	2.10	0.90	3.00	82
	2,220	27	7	15.5	2.10	0.83	2.93	82
	2,220	27	7	15.5	2.10	0.93	3.03	82
N.C.	2,070	30	10	20.7	3.07	0.62	3.69	70
	2,210	31	10	22.1	3.37	0.55	3.92	73
	2,070	30	10	20.7	3.12	0.59	3.71	70
J.W.	2,000	29	10	20.0	2.93	1.02	3.95	68
	1,840	30	13	24.0	3.55	1.31	4.86	62
	1,980	29	10	19.8	2.90	1.22	4.12	68
G.S.	1,880	28	13	24.0	3.38	1.01	4.39	68
	1,840	28	13	24.0	3.38	0.87	4.25	67
	1,900	28	13	24.7	3.47	1.50	4.97	68
Mean	2,090	29	10	20.5	3.04	0.95	3.99	72
SE				± 0.65	±0.16	±0.09	±0.20	± 2

* The distensibility of the entire respiratory apparatus, including lungs and chest bellows. Pt = intrapulmonic pressure at the end of the tidal volume; f = respiratory frequency; Ve = minute ventilation.

celeration is increased, being 80 cc per cm of H_2O (2.1 per cent of vital capacity per cm of H_2O) at 1 G and 52 cc per cm of H_2O (1.3 per cent vital capacity per cm of H_2O at 4 G). The static 1 G total pulmonary compliance agrees roughly with that reported by other investigators (5, 14, 15).

3. Total pulmonary work of breathing. Tidal volume, work of breathing and total dynamic pulmonary compliance are listed for each subject at both 1 and 4 G in Tables II and III, respectively.

Minute ventilation was constant at both accelerations. However, in order to maintain this constant and continue to be adequately ventilated during acceleration, it was found necessary to use large tidal volumes and hence minute ventilations. Since this resulted in abnormally high values for

TABLE III

*The work of breathing and total dynamic compliance * at 4 G*

Subject	Tidal	Pt	f	Ve	Work of breathing			Dynamic compliance
					Elastic	Non-elastic	Total	
	cc	*cm H₂O*		*L/min*		*kg/m/min*		*cc/cm H₂O*
J.F.	1,210	29	17	20.6	5.20	1.03	6.23	42
	1,280	29	17	21.8	5.55	0.84	6.39	44
	1,440	29	17	24.5	6.24	0.51	6.75	49
N.C.	840	30	24	20.2	8.23	0.51	8.74	28
	950	30	24	22.8	9.30	0.26	9.56	32
	950	31	24	22.8	9.30	0.39	9.69	31
J.W.	1,120	31	20	22.4	7.06	1.21	8.27	36
	1,120	31	20	22.4	7.06	1.13	8.19	36
	1,000	31	18	18.0	5.65	0.94	6.59	33
G.S.	1,020	30	16	16.3	3.87	0.77	4.64	34
	1,020	30	16	16.3	3.87	0.77	4.64	34
	1,170	30	16	18.7	4.47	0.77	5.24	39
Mean	1,093	30	19	20.6	6.32	0.76	7.08	37
				± 0.8	±0.56	±0.08	±0.52	± 2

* See footnote to Table II.

mechanical work (11), the increment of work between 1 and 4 G rather than the absolute value is meaningful.

The total work of breathing approximately doubled from 1 to 4 G, with the increase being entirely in the "elastic work" component. There was no change in non-elastic work.

The total dynamic pulmonary compliance decreased from 1 to 4 G. This change was highly significant by the method of significance of differences between means (13).

DISCUSSION

It has become evident that with the accelerations necessary for successful space flight, the subject must be oriented so that the inertial effect is transverse to the long axis of the body. In this position the anticipated rocket acceleration patterns for manned space vehicles are tolerable by humans. Indefinite tolerance to forward acceleration, however, is limited by respiratory embarrassment (1). It has been shown that the respiratory defect during transverse acceleration is primarily restrictive since the maximal breathing capacity and 0.5 second vital capacity decrease less rapidly than does the total vital capacity (2). It has also been demonstrated that oxygen consumption increases (16). Therefore, it appears that forward acceleration from a mechanical aspect applies an added load upon the muscles of respiration, decreasing lung volumes and increasing work without evidence of bronchial obstruction. The experiments reported in this paper offer further evidence for this concept.

As acceleration increases, intrapulmonic pressure increases for a constant lung volume. The increase is the same for each G regardless of the lung volume (Figure 4). This implies that while breathing ambient air the respiratory pump is working against an additional resistance which, in our studies, at 4 G is equivalent to approximately 15 mm of Hg. One might describe this accelerative force by saying that it effectively increases the weight of the thorax and lung which in turn compresses the volume of air within the alveoli. We have elected to term this force PG since it appears to be a constant for each level of acceleration. Rahn and co-workers (5) analyzed the relaxation pressure (Pr) as the algebraic sum of two fractions, the lung elasticity (Pl) and the chest elasticity (Pc) where the latter includes all of the elastic or gravitational factors other than those of the lung itself. During acceleration the relaxation pressure at each G is greater than the resting relaxation pressure by an amount equal to PG.

Non-elastic work between 1 and 4 G does not change. However, because of the additional resistance to inspiration offered by forward acceleration (PG), "elastic" and total work of breathing double. The change in compliance adds only a small fraction of this increase in "elastic" work. In other experiments, it has been observed that respiratory frequency increases while tidal volume decreases during increasing forward acceleration (16). This probably represents the optimal combination for minimizing the increase in the work of breathing (11, 17). The cause of the increased oxygen requirement during acceleration described by two of the authors (16) is in all probability partly the result of this increased work.

The static total pulmonary compliance for each G over the same volume range is the same, indicating that there is no definite change in the overall elastic properties of the thorax and lung. However, if the static compliance is measured from an end-expiratory position, it progressively diminishes with increasing acceleration. Since the relaxation pressure-volume curves (Figure 4) are parallel, this decrease is the result of the fall in functional residual capacity (FRC) and pulmonary mid-position (18–19). Dynamic compliance also decreases during acceleration and probably for the same reason. Christie (20) and others (21, 22) have shown that compliance decreases with increasing respiratory frequency when airway resistance is increased. However, we have no evidence of increased airway resistance during acceleration since there is no change in non-elastic work or timed vital capacity.

During 4 G forward acceleration the relaxation pressure (Figure 4) even at zero lung volume is positive. This was true regardless of the method used. The reasons for this are obscure. It is possible that blood pools into the pulmonary circuit upon relaxation after a forced expiration, or that during a forced expiration under acceleration there is air-trapping within alveoli, which is released upon relaxation. Regardless of the cause, it implies that inspiration during 4 G or greater for-

ward acceleration is always active, regardless of the end-expiratory position, and complete relaxation of the inspiratory muscles does not occur.

Respiratory frequency during forward acceleration increases linearly at least up to 12 G (16). Since forward acceleration resembles chest compression, the increase in frequency may in part be due to stretch or proprioceptive reflexes. Culver and Rahn (23) have demonstrated that chest compression by pneumatic cuff or Drinker respirator results in a decrease in tidal volume and an increase in respiratory frequency, both of which can be nullified by vagal blocking.

All of the lung volumes decrease proportionately during forward acceleration, with the exception of residual volume. The largest percentage of reduction is in the expiratory reserve volume. This implies a downward shift of the mid-pulmonary position, and a decrease in the FRC. Agostoni, Thimm and Fenn (24) considered the FRC as a buffer zone for maintenance of alveolar composition. They demonstrated that as the FRC decreased, respiratory frequency increased, maintaining within small limits changes in the composition of alveolar air. This relationship between respiratory frequency and FRC is evident during forward acceleration (16) and would theoretically minimize fluctuations in alveolar gas composition and rate of work.

Residual volume between 1 and 4 G does not significantly change. This is surprising, since it was expected that during forward acceleration residual volume would decrease as the result of thoracic compression and increase in pulmonary blood volume. Changes in residual volume secondary to shifts in pulmonary blood volume have been demonstrated by Fowler, Guillet and Rahn (7) using continuous positive or negative pressure breathing in a Drinker respirator. However, these changes were small, and the degree of negative pressure breathing that produced a significant change far exceeded that which would be equivalent to 4 G acceleration. During acceleration, residual volume becomes a larger percentage of total lung capacity. Whether or not this increased ratio is significant enough to be a factor in altering gas exchange remains to be evaluated. This pattern of lung volumes is the same as that seen in other situations of thoracic constriction

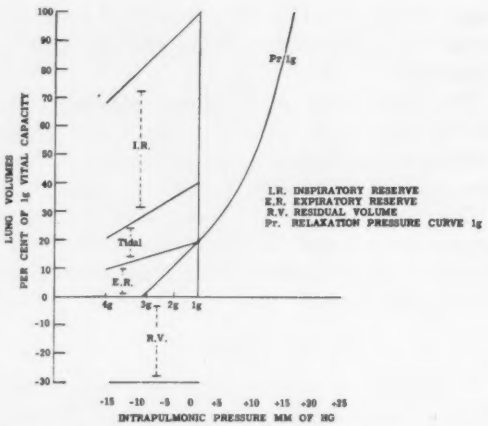

FIG. 5. PRESSURE-VOLUME DIAGRAM OF THE CHEST DURING INCREASING FORWARD ACCELERATION.

such as kyphoscoliosis and after encasing the thorax of a normal individual in a restricting corset (25, 26).

The result described here support the hypothesis that mechanically forward acceleration resembles negative pressure breathing. This may be clarified if all the existing information is combined in one diagram, using as a model the pressure-volume diagram of the chest described by Rahn and associates (5). They constructed the diagram from information obtained in relaxation pressure-volume curves, and lung volume changes as the result of negative and positive pressure breathing. We can construct a similar diagram (Figure 5) using the same information by assuming that PG at various accelerations is equivalent to a pressure applied to the body in a manner analogous to the pressure applied to the body by a Drinker respirator during negative pressure breathing. Therefore, we can place the 4 G lung volumes at a negative 15 mm of Hg, and the 1, 2 and 3 G lung volumes at their respective pressures.

It is not meant to imply that all the abnormalities occurring in respiratory physiology during forward acceleration are the result of negative pressure breathing, as inequalities of perfusion, diffusion and ventilation may be present. However, this study provides a basis for the concept that positive pressure breathing may reverse the abnormalities of respiratory mechanics and increase the tolerance to forward acceleration.

SUMMARY

The respiratory mechanics of forward acceleration were studied in four normal subjects. All of the lung volumes decreased proportionately with the exception of residual volume which became a larger fraction of the total lung capacity. The resting mid-pulmonary position decreased. As acceleration increased, intrapulmonic pressure increased for a constant volume, shifting the relaxation pressure-volume curve downward and to the right. Static and dynamic total pulmonary compliances decreased as a result of the decrease in functional residual capacity. Elastic work approximately doubled due to the work necessary to overcome acceleration resistance (PG). Nonelastic work did not change. Inspiration during 4 G acceleration was always active regardless of the end-expiratory position.

It is suggested that respiratory mechanics during forward acceleration and negative pressure breathing are similar.

REFERENCES

1. Clarke, N. P., Bondurant, S., and Leverett, S. D. Human tolerance to prolonged forward and backward acceleration. Aerospace Med. 1959, **30**, 1.
2. Cherniack, N. S., Hyde, A. S., and Zechman, F. W., Jr. Effect of transverse acceleration on pulmonary function. J. appl. Physiol. 1959, **14**, 914.
3. Hershgold, E. J. Roentgenographic study of human subjects during transverse accelerations. Aerospace Med. 1959, **31**, 213.
4. Holt, J. P. The effect of positive and negative intrathoracic pressure on cardiac output and venous pressure in the dog. Amer. J. Physiol. 1944, **142**, 594.
5. Rahn, H., Otis, A. B., Chadwick, L. E., and Fenn, W. O. The pressure-volume diagram of the thorax and lung. Amer. J. Physiol. 1946, **146**, 161.
6. Mills, J. N. The influence upon the vital capacity of procedures calculated to alter the volume of blood in the lung. J. Physiol. (Lond.) 1949, **110**, 207.
7. Fowler, R. C., Guillet, M., and Rahn, H. Lung volume changes with positive and negative pulmonary pressures *in* Studies in Respiratory Physiology, Air Force Technical Report no. 6528, August, 1951, p. 522.
8. Hamilton, W. F., and Mayo, J. P. Changes in the vital capacity when the body is immersed in water. Amer. J. Physiol. 1944, **141**, 51.
9. Lundsgaard, C., and Van Slyke, D. D. Studies of lung volume. I. Relation between thorax size and lung volume in normal adults. J. exp. Med. 1918, **27**, 65.
10. Rahn, H., Fenn, W. O., and Otis, A. B. Daily variations of vital capacity, residual air, and expiratory reserve including a study of the residual air method. J. appl. Physiol. 1949, **1**, 725.
11. Otis, A. B., Fenn, W. O., and Rahn, H. Mechanics of breathing in man. J. appl. Physiol. 1950, **2**, 592.
12. McIlroy, M. B., Marshall, R., and Christie, R. V. The work of breathing in normal subjects. Clin. Sci. 1954, **13**, 127.
13. Mainland, D. Elementary Medical Statistics. Philadelphia, W. B. Saunders, 1952, p. 149.
14. Nims, R. G., Conner, E. H., and Comroe, J. H., Jr. The compliance of the human thorax in anesthetized patients. J. clin. Invest. 1955, **34**, 744.
15. Butler, J., and Smith, B. H. Pressure-volume relationships of the chest in the completely relaxed anaesthetised patient. Clin. Sci. 1957, **16**, 125.
16. Zechman, F., Jr., Cherniack, N. S., and Hyde, A. S. Ventilatory response to forward acceleration. Fed. Proc. 1960, **19**, 376.
17. Cook, C. D., Sutherland, J. M., Segal, S., Cherry, R. B., Mead, J., McIlroy, M. B., and Smith, C. A. Studies of respiratory physiology in the newborn infant. III. Measurements of mechanics of respiration. J. clin. Invest. 1957, **36**, 440.
18. Nisell, O. I., and DuBois, A. B. Relationship between compliance and FRC of the lungs in cats, and measurements of resistance to breathing. Amer. J. Physiol. 1954, **178**, 206.
19. Cook, C. D., Helliesen, P. J., and Agathon, S. Relation between mechanics of respiration, lung size, and body size from birth to young adulthood. J. appl. Physiol. 1958, **13**, 349.
20. Christie, R. V. The elastic properties of the emphysematous lung and their clinical significance. J. clin. Invest. 1934, **13**, 295.
21. Otis, A. B., McKerrow, C. B., Bartlett, R. A., Mead, J., McIlroy, M. B., Selverstone, N. J., and Radford, E. P. Mechanical factors in distribution of pulmonary ventilation. J. appl. Physiol. 1955, **8**, 427.
22. Mead, J., Lindgren, I., and Gaensler, E. A. The mechanical properties of the lung in emphysema. J. clin. Invest. 1955, **34**, 1005.
23. Culver, G. A., and Rahn, H. Reflex respiratory stimulation to chest compression in the dog *in* Studies in Respiratory Physiology, Air Force Technical Report no. 6528, August, 1951, p. 78.
24. Agostoni, E., Thimm, F. F., and Fenn, W. O. Comparative features of mechanics of breathing. J. appl. Physiol. 1959, **14**, 679.
25. Bergofsky, E. H., Turino, G. M., and Fishman, A. P. Cardiorespiratory failure in kyphoscoliosis. Medicine (Baltimore) 1959, **38**, 263.
26. Caro, C. G., Butler, J., and DuBois, A. B. Some effects of restriction of chest cage expansion on pulmonary function in man: An experimental study. J. clin. Invest. 1960, **39**, 573.

ESOPHAGEAL APERISTALSIS AND ACHALASIA PRODUCED IN DOGS BY PROLONGED CHOLINESTERASE INHIBITION *

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Considerable evidence suggests that cardiospasm, or achalasia of the esophagus, is a disease of the intrinsic innervation of that organ (1–6). If so, a neurotoxic agent might produce a similar picture in experimental animals. This possibility is enhanced by the report of Koelle and Gilman (7) who, while studying the effects of prolonged administration to dogs of the anticholinesterase agent di-isopropylfluorophosphate (DFP), noted the appearance of objective evidences of dysphagia, esophageal stasis, and "cardiospasm." However, not all motor dysfunctions causing dysphagia and esophageal stasis in man are manifestations of cardiospasm, and no studies have been performed to show that DFP-induced canine "cardiospasm" is indeed analogous to the condition seen in man. Consequently, we undertook to see if *1*) prolonged DFP administration does regularly produce an esophageal motor disorder in dogs, and *2*), if so, to determine its nature and its similarity to human cardiospasm.

METHODS

Six adult mongrel dogs, 2 male and 4 female, weighing 15 to 18 kg, were prepared with permanent cervical esophageal fistulas by the method of Ware and Howe (8). This preparation allows natural feeding with only minimal leakage, yet permits ready insertion of recording balloons, tubes and barium with no discomfort to the unanesthetized animal. Special care was taken during preparation of the fistula to identify and avoid injury to the vagi. All dogs ate and drank normally and maintained their weight before and after the fistulas were prepared. Weights were recorded weekly throughout the control and experimental periods. The dogs were easily trained to lie quietly for as long as 4 hours, unrestrained, while studies were done.

DFP (0.5 per cent in peanut oil) was administered intramuscularly in alternate hind legs three times weekly. Three dosage schedules were used: Dog 1 received 0.5 mg per kg; Dogs 2, 3 and 4, 0.3 mg per kg; and Dogs 5 and 6, 0.2 mg per kg. The total dosage was calculated on the basis of the control weights and was not changed as the weight fluctuated during the experimental period. DFP was discontinued at the onset of severe skeletal muscle weakness or marked esophageal motor abnormalities. Serum and erythrocyte cholinesterase levels were periodically measured by Michel's method (9).

Balloon kymography was performed twice weekly during the control and experimental periods by means of an essentially isobaric system (10). At the first kymography session the balloon was allowed to advance into the stomach and then withdrawn until it was estimated to lie in the distal third of the esophagus. After the balloon's position was checked fluoroscopically, the tube was marked and during each subsequent study was introduced for the same distance. Since small parenteral doses of acetyl-β-methyl choline chloride (methacholine chloride) have been shown to cause a sustained contraction of the lower esophagus in human cardiospasm (11), this agent was administered intramuscularly at weekly intervals during the control and experimental periods to assess its effect on the balloon kymographic record. During the control period it was determined that 0.75 mg of methacholine chloride consistently produced moderate but not excessively severe systemic effects, and this was chosen as the standard dosage.

Cine-fluororadiography, with aqueous barium sulfate suspension as contrast material, was performed during the control and experimental periods. The barium was introduced into the cervical esophagus by way of a soft rubber catheter in the fistula.

In Dogs 3 and 6, intraesophageal pressures in the lower esophagus were studied with open-tipped, water-filled, polyvinyl catheters of 1.4 mm ID. Simultaneous measurements were made from 3 tips spaced 3 cm apart. The tubes contained mercury markers to facilitate positioning at fluoroscopy. Pressure changes were transmitted to Sanborn pressure transducers and graphed on a Sanborn 4-channel recorder.

Microscopic anatomy of the esophagus of 3 normal dogs and of Dogs 1 and 5, which died after receiving DFP for 2 and 4 weeks, respectively, was studied in sections stained with hematoxylin and eosin. All but Dog 1 were autopsied within 30 minutes of death. Particular attention was directed to Auerbach's plexus. Postmortem change in

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size of the esophagus with resultant change in "concentration" of ganglion cells was minimized by measuring the esophagus *in situ* and maintaining it at this size during fixation. After fixation the esophagus was "rolled" so that sections representing the entire length of the esophagus could be encompassed on a single slide.

RESULTS

I. Control period. Balloon kymographic records showed a regular pattern of contractions at a rate of 6 to 8 per minute in 4 dogs. Two animals, however, (Dogs 1 and 3) had a consistently different pattern with the irregular occurrence of periods of inactivity varying in length up to 1 minute. Tho two types of control records are shown in Figure 1. It can be seen that about 20 ml of air enters the balloon before a contraction occurs; and in the essentially isobaric recording system used, this volume may be regarded as an expression of esophageal tonus (10). Both types of control records exhibited similar tonus, the figure obtained also being comparable with that recorded in the normal human esophagus. In all animals the balloon was rapidly propelled into the stomach when the tube was released.

Cine-fluororadiographic examination of the esophagus demonstrated active and effective peristalsis qualitatively similar to that seen in man.

Serum cholinesterase levels ranged from 0.97 to 1.52 Δ pH per hour, erythrocyte cholinesterase levels from 0.08 to 0.17 Δ pH per hour. For the individual animals the values were consistent.

II. Experimental period. After DFP was started, no effects were noted for 3 to 4 weeks, whereupon weakness and incoordination of the hind legs became apparent and progressed to a variable extent. The final degree of paresis varied markedly and bore little relationship to the total amount of DFP given (Table I). Dog 2 developed severe hind leg paresis while Dogs 3 and 4, which received comparable doses, developed only minimal symptoms. About one month after DFP was discontinued, hind leg strength in the surviving dogs gradually began to improve. This recovery was incomplete, however, and the more severely affected dogs had considerable weakness a year later.

Dogs 1, 4 and 5 manifested muscarinic effects of wheezing and dyspnea which were transiently present for about a week in Dog 4, but were noted to death in Dogs 1 and 5. These symptoms, as well as the weakness and incoordination, became much more severe after exercise. Muscular fibrillation was not seen. Transient urinary incontinence was observed in Dog 2, while Dog 1 had urinary and fecal incontinence for 4 to 5 days prior to death. Diarrhea was not observed. Small bowel balloon kymography was performed on Dog 6 eight weeks after DFP was stopped. The record obtained was entirely comparable with a record obtained from a normal dog as well as with normal human records. Although gastric peristalsis and emptying time were not specifically

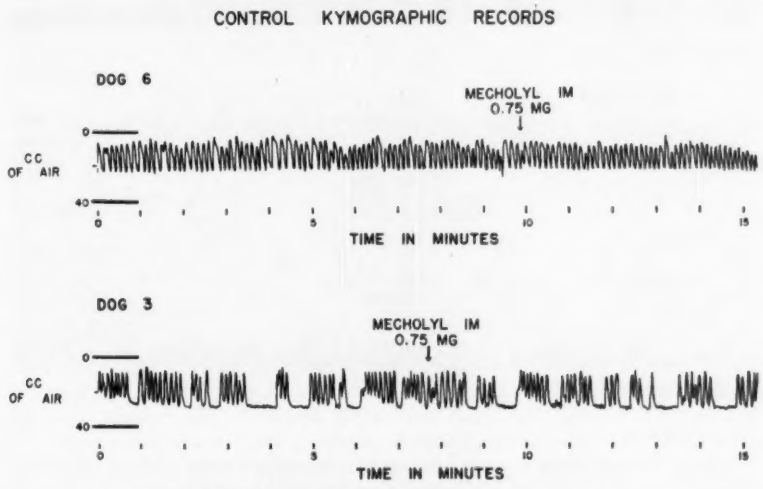

FIG. 1. CONTROL BALLOON KYMOGRAPHIC RECORDS.

TABLE I

Effect of di-isopropylfluorophosphate given three times weekly, in dogs

Dog	Sex	Weight	Single dose	No. of doses to onset of musc. weakness	Total dose to onset of musc. weakness	No. of doses to esoph. paralysis	Total dose to esoph. paralysis	Extent of paresis
		kg	*mg/kg*		*mg/kg*		*mg/kg*	
1	F	18	0.50	6	3.0	9	4.5	Died
2	M	15	0.30	10	3.0	15	4.5	Moderately severe
3	F	15	0.30	13	3.3	17	5.1	Minimal
4	M	19	0.30	13	3.3	17	5.1	Minimal
5	F	15	0.20	13	2.6	19	3.8	Died
6	F	16	0.20	10	2.0	19	3.8	Moderately severe

recorded, no gross change was observed at fluoroscopy.

In all dogs, evidences of dysphagia with regurgitation of food and fluid and resultant weight loss began about one week after the first evidence of hind leg weakness, i.e., four to five weeks after starting DFP. Terminally, Dogs 1 and 5 seemed able to swallow little if anything. In the surviving dogs, swallowing difficulties began to clear about four weeks after DFP was stopped, and their weight gradually returned to near control levels.

Changes in the balloon kymographic records were first noted shortly before the onset of evidences of dysphagia. These early changes consisted of prolonged periods of inactivity and a decreased torus so that about 30 ml of air was allowed to enter the balloon. Coincident with the signs of dysphagia, esophageal muscular activity virtually ceased, i.e., no contractions were observed for a 10 to 15 minute period, or at most no more than 10 irregularly occurring contractions during any 10 minute period. At this time the balloon remained stationary when released instead of being rapidly propelled into the stomach as in the control period. However, the balloon could be pushed into the stomach without difficulty.

With the cessation of esophageal muscular activity, DFP was stopped. Six to seven weeks later random, irregularly occurring waves began to appear. Figure 2 shows the sequence of tracings in a dog with a regular control record, and Figure 3 the sequence in a dog with an irregu-

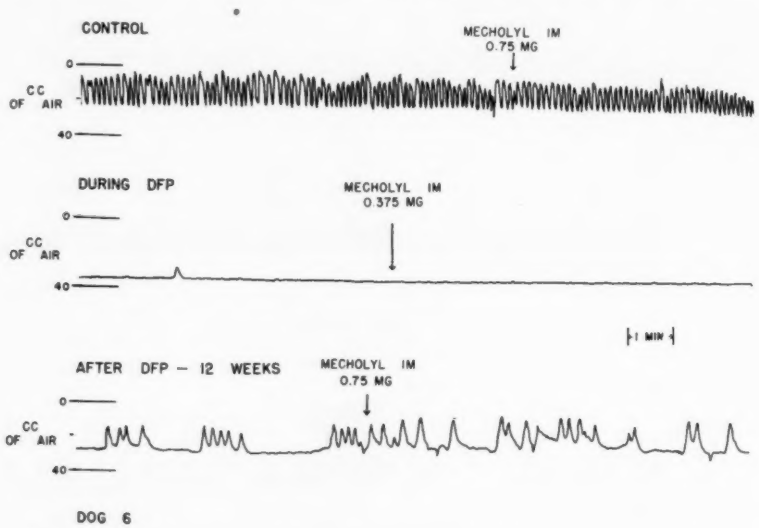

FIG. 2. SEQUENCE OF BALLOON KYMOGRAPHIC RECORDS IN A DOG WITH A REGULAR CONTROL RECORD.

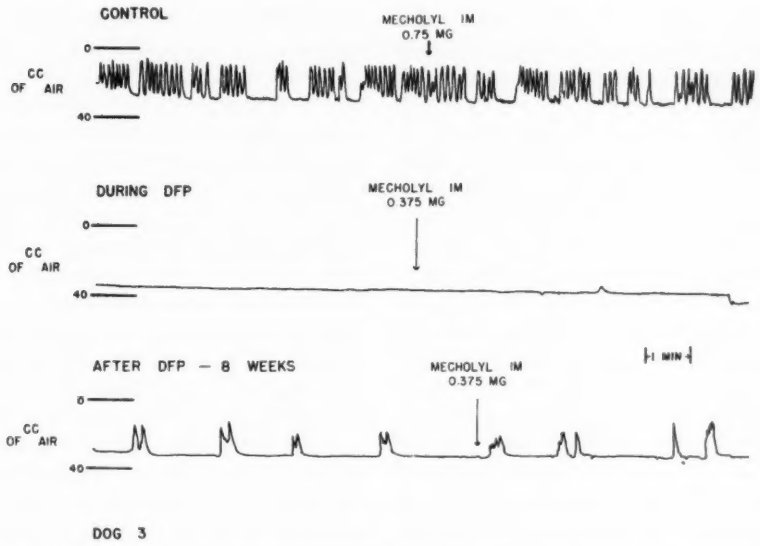

FIG. 3. SEQUENCE OF BALLOON KYMOGRAPHIC RECORDS IN A DOG WITH AN IRREGULAR CONTROL RECORD.

lar control record. Both show greatly decreased tonus during the aperistaltic period with slight increase in tonus when some activity had returned. This sequence occurred in all dogs. In the course of a year esophageal motor activity increased, but did not return to normal.

Intramuscular methacholine chloride produced no esophageal effects during either the control or

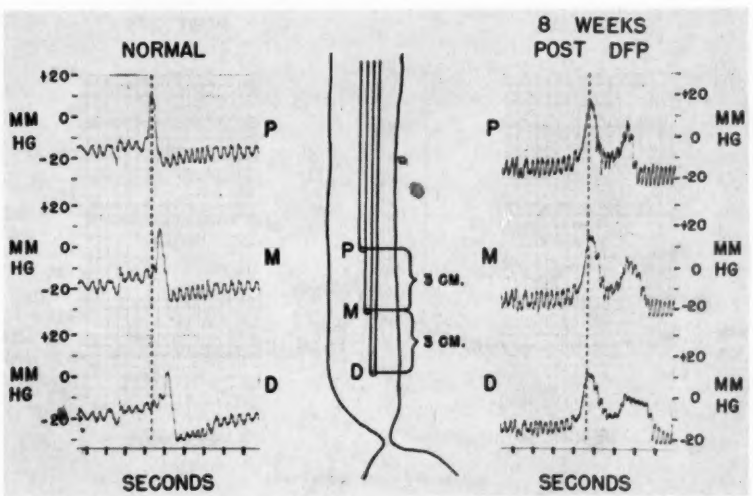

FIG. 4. NORMAL AND POST-DI-ISOPROPYLFLUOROPHOSPHATE (DFP) INTRALUMINAL PRESSURE RECORDS FROM THE DISTAL ESOPHAGUS. Deglutition occurred approximately two seconds before the positive pressure deflection started. Note the progressive peak of pressure (peristalsis) in the normal record as contrasted to simultaneous peaks of pressure recorded 8 weeks after DFP was stopped.

experimental period. While DFP was being given, it became necessary to reduce the dosage of methacholine chloride from the standard 0.75 mg to as low as 0.25 mg to prevent excessively severe systemic reactions. After DFP was stopped, it was possible to increase the dose slowly to control levels. Dog 6 was given 2.0 mg of methacholine chloride on one occasion while she was receiving DFP and on another after DFP was stopped. Each time profuse salivation, hyperpnea, agitation and restlessness, vomiting and defecation appeared, but no esophageal effects were recorded.

Cine-radiographic examination of the esophagus at the peak of DFP effect showed striking changes. Barium suspension introduced through the fistula with the dog horizontal remained pooled in the body of the esophagus and no muscular activity was seen. The addition of more barium suspension did not result in any motor response; the organ merely dilated. To assess the effects of gravity, the animals were then held upright and examined at intervals without the addition of more barium. Invariably, most of the column of barium suspension remained in the esophagus after the animals had been upright for 15 minutes. The esophagus was usually found to be empty after the animals had been held upright for 20 to 30 minutes.

Six to seven weeks after DFP was stopped, when kymographic records showed some return of motor function, the radiologic picture of esophageal dilatation persisted. At this time, however, rapid distention of the esophagus with barium suspension resulted in recurrent segmental contractions, brief in duration, that moved the barium both orally and, to some extent, into the stomach. On occasions these contractions appeared to be distally progressive, but they did not completely occlude the lumen or empty the esophagus. With the passage of these distally progressive but ineffectual waves the barium suspension did not appear to be pushed ahead of the contraction but was merely displaced proximally. This resulted in the interesting picture of a distally progressive contractile wave but proximal or retrograde flow of barium suspension. This retrograde flow occurred even when the animals were in the upright position. In the interval between contractions there were prolonged periods of inactivity, and the esophagus remained filled with barium.

Control intraluminal pressure records showed a distally progressive peak of pressure as a peri-

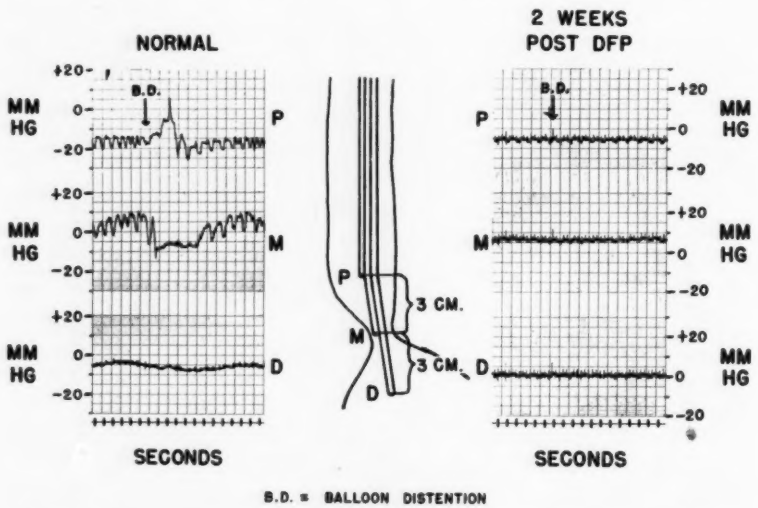

FIG. 5. INTRAESOPHAGEAL PRESSURE RECORDS WITH THE MIDDLE TIP IN THE GASTROESOPHAGEAL SPHINCTERIC AREA. In the normal record, distention of a 40 ml capacity balloon in the upper esophagus or deglutition regularly resulted in a sharp drop of pressure within a second. Although the zone of elevated pressure was found in the recording made two weeks after DFP was stopped, no drop of pressure occurred following balloon distention or deglutition.

staltic wave traveled the esophagus, whether initiated by a dry swallow, a wet swallow, or by distention of a balloon placed in the upper esophagus. This is in contrast to the post-DFP (i.e., recovery) period when peaks of pressure were recorded in all three tips simultaneously (Figure 4). Intraluminal pressures recorded during the height of DFP effect showed only the expected respiratory and cardiac effects without evidence of esophageal muscular activity.

Since the pressure characteristics of the gastroesophageal junctional area had not been investigated in the original group of six, a seventh dog was also given DFP after control pressure studies of the junctional area. In the control period, a zone of elevated pressure which exhibited a sharp drop in pressure in response to deglutition was recorded, findings similar to those previously reported by Schlegel and Code (12). In addition, this drop in pressure could be elicited by rapid distention of a balloon of about 40 ml capacity placed in the upper esophagus (normal, Figure 5). This similarity of response to both deglutition (primary peristalsis) and balloon distention (secondary peristalsis) has previously been reported in the normal human (13). At the height of DFP effect, a similar zone of elevated resting pressure was demonstrated at the gastroesophageal junctional area. However, neither deglutition nor rapid distention of a balloon placed in the upper esophagus resulted in a drop of pressure (post-DFP, Figure 5).

Postmortem examination of the two animals that died during the course of the study showed the esophagus to be dilated but to have no mucosal changes. The remainder of the gastrointestinal tract appeared normal. The number of myenteric ganglion cells of Auerbach's plexus in each of several slides, representing the entire length of esophagus in three normal dogs and the two DFP dogs, was counted. The normal dogs averaged 20, the DFP dogs 15 ganglion cells per slide, an inconclusive difference.

DISCUSSION

A sharp distinction must be drawn between the acute pharmacologic effects of DFP and the residual effects following prolonged administration of the agent. Acutely, DFP phosphorylates and thereby inactivates cholinesterase. The unopposed neurotransmitter acetylcholine thereupon produces continued depolarization of the motor endplate with resultant neuromuscular block, a sequence which has been established particularly for skeletal muscle (14). This acute effect of DFP is usually considered to be completely reversible (14, 15). However, a prolonged and perhaps irreversible motor disorder of the esophagus and hind legs was evident in our dogs long after DFP administration was stopped and persisted in the face of normal serum and erythrocyte cholinesterase levels, i.e., at a time when tissue cholinesterase levels had presumably returned to normal. This effect of prolonged cholinesterase inhibition has been noted in experimental animals (7, 16) and also following long continued exposure to anticholinesterases in man (17–19). It would thus appear that either prolonged excess of the neurotransmitter substance induced by the anticholinesterase agent or the anticholinesterase agent itself may permanently damage some link in the chain of neuromuscular events. Whether the site of this damage is the same as the site of the acute effect is unknown.

The esophageal disorder produced in dogs following prolonged DFP administration resembles cardiospasm in man in several respects. At the height of DFP effect, the esophagus is dilated, peristalsis is absent, the lower esophageal sphincter does not relax in response to either a swallow or balloon distention of the upper esophagus, and residues of barium suspension remain in the esophagus in the face of gravity. Thus the dogs exhibit two cardinal features of human cardiospasm: aperistalsis and failure of relaxation ("achalasia") of the gastroesophageal sphincter. A crucial difference exists, however, between spontaneous human cardiospasm and the motor abnormalities following prolonged DFP administration. In human cardiospasm, exhibition of a small dose of methacholine is followed by a strong, nonpropulsive contraction of the esophagus, but no esophageal effects are noted in the dogs exposed to DFP.

One explanation for the lack of response to methacholine noted in our dogs might be that cardiospasm and prolonged DFP administration

affect neuromuscular transmission at different sites. If present concepts of the nature of cardiospasm are correct, the basic lesion is a poverty of normal ganglion cells of Auerbach's plexus amounting to a "denervation" of the esophagus (1, 3, 6). The muscle cells presumably remain normally polarized and therefore sensitive to depolarizing agents. Since the neuromuscular block produced by acute DFP intoxication is ascribed to an excess of acetylcholine, it is not surprising that the addition of another depolarizing agent in the form of methacholine during the period of DFP administration fails to elicit a contraction. Unfortunately, such a tidy neurochemical difference cannot be drawn between cardiospasm and the condition seen following prolonged DFP administration, but if the block in this condition is also due to failure of repolarization of the motor end-plate, then methacholine would not be expected to result in contraction.

Another factor that might alter the results of prolonged DFP administration is the different structure of the esophagus in man and dog; although similarly innervated, the human esophagus contains predominantly smooth, the canine esophagus predominantly skeletal, muscle (20). How important this histologic difference might be, however, is difficult to assess. Regardless of its muscular morphology, the canine esophagus possesses an innervation typical of intestinal smooth muscle with a myenteric plexus extending over its entire length. Moreover, as judged by balloon kymograms and measurements of peristaltic and lower esophageal sphincter activity, the canine esophagus appears to function in a very similar fashion to the human esophagus. From a functional point of view, therefore, the striated canine esophagus appears to be more comparable with smooth muscle (as in the human esophagus) than with skeletal muscle (as in the gastrocnemius). One is tempted to use the difference in musculature to explain why we observed marked esophageal effects following prolonged DFP intoxication without obvious changes in the remainder of the gastrointestinal tract. However, anticholinesterase agents, including DFP, have been shown to affect the smooth muscled gastrointestinal tract of experimental animals (7, 21) and also man (14, 15, 22).

Other attempts to produce experimental cardiospasm in animals have depended upon either a direct surgical attack on the esophagus or its nerve supply (23, 24) or the injection of a sclerosing agent into the esophageal wall (25). The significance of such procedures is difficult to assess; however, since gross cicatricial or inflammatory effects are also necessarily produced. In addition, the dynamics of the resultant esophageal disorders have not been analyzed in detail nor has the response to methacholine been determined. The major implication of our study is that a severe esophageal motor dysfunction with two features typical of human cardiospasm can be produced experimentally by an agent known to affect neuromuscular transmission. Moreover, this motor abnormality can be produced without concomitant gross or even microscopic change in esophageal structure. It is therefore possible that a condition resembling cardiospasm in all respects, or even the condition in man itself, may be produced by a neurotoxin that affects the proper site in the sequence of neuromuscular tansmission.

SUMMARY

The prolonged administration of the anticholinesterase agent di-isopropylfluorophosphate to dogs resulted in *1*) esophageal aperistalsis, and *2*) failure of relaxation of the lower esophageal sphincter in response to swallowing and esophageal balloon distention. These effects persisted for months after the administration of di-isopropylfluorophosphate was stopped. While the esophageal disorder produced resembled human cardiospasm, the characteristic response of the human cardiospastic esophagus to methacholine chloride was lacking. It is postulated that difference in site of neurochemical lesion could account for this discrepancy and that neurotoxins acting at somewhat different sites could reproduce the picture of spontaneous human cardiospasm in its entirety.

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REFERENCES

1. Lendrum, F. C. Anatomic features of cardiac orifice of the stomach, with special reference to cardiospasm. Arch. intern. Med. 1937, **59**, 474.
2. Rake, G. W. On the pathology of achalasia of the cardia. Guy's Hosp. Rep. 1927, **77**, 141.
3. Cross, F. S. Pathologic changes in megaesophagus (esophageal dystonia). Surgery 1952, **31**, 647.
4. Etzel, E. Megaoesophagus and its neuropathology; clinical and anatomo-pathological research. Guy's Hosp. Rep. 1937, **87**, 158.
5. Cameron, J. A. Oesophagectasia in a child. Arch. Dis. Childh. 1927, **2**, 358.
6. Kramer, P., and Ingelfinger, F. J. Cardiospasm, a generalized disorder of esophageal motility. Amer. J. Med. 1949, **7**, 174.
7. Koelle, G. B., and Gilman, A. Chronic toxicity of di-isopropylflurophosphate (DFP) in dogs, monkeys and rats. J. Pharmacol. exp. Ther. 1946, **87**, 435.
8. Ware, P. F., and Howe, C. W. Experimental esophageal and pharyngocervical fistula. Arch. Surg. (Chicago) 1951, **63**, 229.
9. Michel, H. O. An electrometric method for the determination of red blood cell and plasma cholinesterase activity. J. Lab. clin. Med. 1949, **34**, 1564.
10. Kramer, P., and Ingelfinger, F. J. Motility of the human esophagus in control subjects and in patients with esophageal disorders. Amer. J. Med. 1949, **7**, 168.
11. Kramer, P., and Ingelfinger, F. J. Esophageal sensitivity to mecholyl in cardiospasm. Gastroenterology 1951, **19**, 242.
12. Schlegel, J. F., and Code, C. F. Pressure characteristics of the esophagus and its sphincters in dogs. Amer. J. Physiol. 1958, **193**, 9.
13. Fleshler, B., Hendrix, T. R., Kramer, P., and Ingelfinger, F. J. The characteristics and similarity of primary and secondary peristalsis in the esophagus. J. clin. Invest. 1959, **38**, 110.
14. Grob, D. The manifestations and treatment of poisoning due to nerve gas and other organic phosphate anticholinesterase compounds. A.M.A. Arch. intern. Med. 1956, **98**, 221.
15. Grob, D., and Harvey, J. C. Effects in man of the anticholinesterase compound Sarin (isopropyl methyl phosphonofluoridate). J. clin. Invest. 1958, **37**, 350.
16. Hunt, C. C., and Riker, W. F., Jr. The effect of chronic poisoning with di-isopropyl fluorophosphate on neuromuscular function in the cat. J. Pharmacol. exp. Ther. 1947, **91**, 298.
17. Petty, C. S. Organic phosphate insecticide poisoning. Residual effects in two cases. Amer. J. Med. 1958, **24**, 467.
18. Bidstrup, P. L., Bonnell, J. A., and Beckett, A. G. Paralysis following poisoning by a new organic phosphorus insecticide (Mipafox): Report on 2 cases. Brit. med. J. 1953, **1**, 1068.
19. Glasson, J., and Stelling, F. H. An unusual cause for paralysis of the extremities. Sth. med. J. (Bgham, Ala.) 1956, **49**, 1325.
20. Ingelfinger, F. J. Esophageal motility. Physiol. Rev. 1958, **38**, 533.
21. Lindenberg, R. Morphologic alterations in the spinal cord of cats after subacute exposure to DFP (diisopropylphosphofluridate). Chemical Corps Medical Laboratory Research Report, no. 235.
22. Walsh, A. DFP in the treatment of paralytic ileus. Irish J. med. Sci. 1957, no. 377, 241.
23. Agrifoglio, M. Ricerche sperimentali sulla dilatazione diffusa dell' esofago. Arch. ital. Chir. 1931, **28**, 337.
24. Hwang, K., Essex, H. E., and Mann, F. C. A study of certain problems resulting from vagotomy in dogs with special reference to emesis. Amer. J. Physiol. 1947, **149**, 429.
25. Deloyers, L., Cordier, R., and Duprez, A. A new approach to the physiology of so-called cardiospasm: Experimental production of cardiospasm in cats after destruction of Auerbach's plexus. Ann. Surg. 1957, **146**, 167.

PROTEINPOLYSACCHARIDES OF HUMAN COSTAL CARTILAGE *

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From bovine nasal cartilage a product has been isolated which seemed to behave as a compound and which contained chondroitin sulfate and protein; this product was called chondromucoprotein (1). In similar work with human costal cartilage, greater difficulty was encountered. Although a somewhat analogous product was isolated, its characterization as a chemical entity was far less satisfactory, since its composition seemed to vary rather widely in the proportions of protein and polysaccharide present in samples prepared from the cartilage of different individuals. Bovine nasal chondromucoprotein, during the course of many scores of preparations, seemed to be constant in composition. Recently it was found that bovine nasal chondromucoprotein could be fractionated centrifugally to give two distinct products, each consisting of both protein and polysaccharide (2). These products were called proteinpolysaccharides and were distinguished as heavy and light, depending upon their sedimentation properties. They were referred to as PP-H and PP-L, and were found to consist of very different proportions of protein and polysaccharide. The present report describes the isolation from human costal cartilage of a crude product analogous to bovine nasal chondromucoprotein, describes attempts to arrive at products of constant composition by repeated reprecipitation, and then describes the ultracentrifugal separation of the crude material into two fractions analogous to the PP-H and PP-L of bovine nasal cartilage but not identical with them.

Since use of the word mucoprotein is to be discontinued it will be best to discontinue use of the word chondromucoprotein also and to refer to the product it described as a crude proteinpolysaccharide (crude PP). Where it is necessary to distinguish between products isolated from bovine nasal cartilage and those isolated from human costal cartilage, the letters BN or HC will be set befor the product. Then the products separated from crude HC-PP by ultracentrifugation can be called HC-PP-L and HC-PP-H.

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EXPERIMENTAL METHODS

Extraction procedure. Human costal cartilage was collected about 24 to 48 hours after death and cleaned in a cold room to remove all noncartilaginous tissue. It was diced into pieces about 6 mm on a side and all grossly calcified pieces were discarded. Weighed samples were dried by beating about 15 g at a time in a Waring blendor with ethanol (250 ml) for 3 minutes in a cold room. The finely shredded product was transferred to a centrifuge cup, washed by centrifuging with ethanol and absolute ether and dried *in vacuo*. In columns 3, 4 and 5 of Table I are values for the dry weights as fractions of the wet weights, and the nitrogen and hexosamine (against glucosamine as standard) contents of the dried samples from 19 individuals varying in age from 7 to 74 years. There are no obvious differences in the dry weights or in the nitrogen contents with either sex or age. A possible trend in hexosamine content with age is suggested, since all values above 7 per cent occur at ages less than 34 and all values below 6 per cent occur at ages over 56.

After preliminary experiments had shown that a crude HC-PP could be extracted from human rib cartilage, it seemed desirable to learn whether it could be extracted as readily from alcohol-dried cartilage as from fresh wet cartilage, since this knowledge would facilitate further comparative studies. Each of the fresh wet cartilage samples represented in Table I was divided into two parts; one part was worked up directly to extract the crude HC-PP from the fresh wet cartilage, the second part was first dried as described above and then worked up to extract its crude HC-PP.

All extraction operations were run in a cold room at 5° C. For each extraction an amount of cartilage was used corresponding to 10 to 20 g of dry cartilage. When wet cartilage was used, yields were calculated on the basis of the dry cartilage weight as given in column 3 of Table I. Either dried cartilage (4 g) or fresh wet cartilage (8 g) was beaten with water (280 ml) in a VirTis-45 homogenizer at its top speed for 30 minutes in a flask (500 ml) with baffles set in an ice bath. To the creamy homogenate was added ethanol (560 ml) and the mixture was centrifuged (4,700 rpm) for one-half hour. The supernatant solution was filtered through a

TABLE I

Data on human costal cartilage and the crude HC-PP isolated from it *

Age	Sex	Cartilage			Crude HC-PP from fresh wet cartilage			Crude HC-PP from dried cartilage		
		Dry wt / Wet wt	N	Hexosamine	Yield	N	Hexosamine	Yield	N	Hexosamine
yrs			%	%	*g/g cartilage*	%	%	*g/g cartilage*	%	%
7	M	0.38	12.3	6.20	0.15	5.80	14.8	0.14	5.10	14.4
20	F	0.51			0.14	7.20	14.0	0.25	7.30	14.1
21	F	0.37	10.8	6.60	0.28	6.76	13.3	0.22	5.80	13.8
21	M	0.41	12.2	7.55	0.23	7.38	15.5	0.28	6.80	14.7
25	F	0.46	11.1	8.67	0.19	7,74	10.4	0.25	7.90	12.8
31	M	0.36	11.5	7.32	0.28	7.85	14.1	0.21	8.00	13.8
34	M	0.44	12.1	6.88	0.24	8.41	12.1	0.20	9.20	11.6
36	M	0.32	12.0	6.86	0.29	8.65	11.5	0.18	9.46	11.7
36	F	0.43	10.9	6.90	0.22	9.30	12.0	0.21	9.30	12.0
40	F	0.51		6.04	0.14	8.71	11.1	0.12	9.60	12.2
43	F	0.48	12.2	6.10	0.21	8.20	11.2	0.17	8.60	10.7
43	F	0.44	11.7	6.75	0.17	9.20	12.1	0.23	9.06	10.8
44	F	0.44	11.5	6.95	0.23	8.53	11.2	0.21	9.01	11.2
45	M	0.41	10.0	6.80	0.26	6.07	10.1	0.27	8.84	10.2
56	M	0.45		6.01	0.27	9.20	10.4	0.24	9.60	10.4
59	F	0.40	11.4	5.54	0.23	9.01	8.5	0.17	9.30	9.9
64	M	0.46	10.8	4.80	0.13	8.29	9.2	0.18	9.56	9.5
69	M	0.40	11.7	5.45	0.21	9.62	9.5	0.20	10.10	8.6
74	M	0.58	11.9	5.72	0.13	8.83	8.8	0.22	9.34	8.4
Average		0.43			0.21			0.21		
Bovine coastal cartilage		0.38	8.2	5.5				0.17	5.7	16.0
Bovine nasal cartilage		0.24	8.3	9.4				0.35	5.2	17.1

* See first page of text for definitions.

plug of glass wool. The residue at this stage is called *R* and is referred to later. It may be dried with alcohol and ether. The filtered extract was opalescent, and on addition of potassium acetate (1 per cent, wt/vol) produced a flocculent precipitate, the crude HC-PP. It was centrifuged and washed with ethanol and ethyl ether and dried in vacuum. Combined yields of the cartilage residue and crude HC-PP amounted to 90 to 95 per cent of the dry weight of the cartilage used.

In Table I, columns 6 and 9 give the yield of crude HC-PP from the fresh wet and from the alcohol-dried cartilage, columns 7 and 10 give their nitrogen content, and columns 8 and 11 give their hexosamine content (against glucosamine as standard). Although there are often great differences in the yield of the product from the wet and the dry cartilage, these seem to be chance fluctuations and the average yields of the product from wet and dry cartilage are identical, 21 per cent of the dry weight of the cartilage used. This is about two-thirds the amount of BN-PP isolated from bovine nasal cartilage by the same procedure. It seems clear that, as with bovine nasal cartilage, drying the human costal cartilage with ethanol before extraction with water makes no difference in the yield of crude HC-PP obtainable. Nor does drying seem to affect the average hexosamine content of the crude products. Nitrogen values of crude products from dried cartilage average slightly higher than those from fresh wet cartilage, but this is probably not significant. The spread of values for both nitrogen and hexosamine content is higher than the spread in corresponding values for BN-PP (1). As with the hexosamine content of the whole cartilage (column 5 of Table I), there seems to be a trend of the hexosamine content of the crude HC-PP to decrease with age (columns 8 and 11). All hexosamine values over 12 per cent occur in products from individuals at ages below 34 and all hexosamine values below 10 per cent occur in products from individuals at ages over 56.

At the bottom of Table I are two rows of figures included for comparative purposes, giving data on bovine cartilage (BC), both costal and nasal, and on the crude BC-PP and BN-PP isolated from them by the same procedure as that described above. It appears that only in the youngest age range does the crude HC-PP approach in composition that of the crude BC-PP or the crude BN-PP.

Reprecipitation procedure. It seemed possible that the fluctuations and the apparent trend in composition with age of the crude HC-PP might be associated with protein impurities. Attempts were made to purify the crude HC-PP by reprecipitation. Each of the samples of crude HC-PP was dissolved in water (about 1 g per 100 ml), any insoluble material was removed by centrifugation, and the proteinpolysaccharide in solution was reprecipitated by addition of potassium acetate to about 2 per cent (wt/vol) and of ethanol to 70 per cent (vol/

TABLE II

Data on crude and reprecipitated HC-PP isolated from human costal cartilage of subjects in the age ranges shown *

	Crude HC-PP			HC-PP once reprecipitated			HC-PP; ratios, $\frac{\text{nitrogen}}{\text{hexosamine}}$ after successive reprecipitations		
	Yield	N	Hexosamine	Yield	N	Hexosamine	Once	Twice	Thrice
yrs	*g/g cartilage*	%	%	*g/g crude HC-PP*	%	%			
7–31	0.22	7.0	13.8	0.74	6.7	14.3	5.9	5.0	5.4
34–56	0.22	8.8	11.3	0.61	8.1	12.9	8.0	7.4	7.7
59–74	0.19	9.2	9.1	0.36	7.9	13.5	7.4	7.7	7.8
Bovine nasal cartilage	0.35	5.2	17.1	0.90	5.3	17.4	3.9	3.9	3.9

* See first page of text for abbreviations.

vol). The reprecipitated product was washed with ethanol and ether, dried, weighed, and analyzed. Table II (columns 5, 6 and 7) summarizes the results, which are given as average yields and as average nitrogen and hexosmaine percentages within each of the age ranges indicated in column 1. The reason for this is that in a number of cases samples were pooled in order to get enough material for the successive reprecipitations. Pooling was done only with samples within 4 years of each other. In columns 2, 3 and 4 are given for comparison the average values of yield, and nitrogen and hexosamine content of the crude HC-PP, already described in Table I and averaged for each of the three age groups indicated in column 1, Table II. Column 5 gives the average yields of the once-reprecipitated HC-PP, and columns 6 and 7 give its nitrogen and hexosamine contents. It is apparent that with advance in age groups there is a drop in the yield of HC-PP obtained on reprecipitation (column 5), and the nitrogen and hexosamine contents of the latter are more nearly uniform in the different age groups. Columns 8, 9 and 10 show the results of further successive reprecipitations. Instead of nitrogen and hexosamine contents there are given molar nitrogen to hexosamine ratios. These do not change significantly with successive reprecipitations after the first. There does, however, persist a difference between the younger age group (7 to 31 years) and the older age group (34 to 74 years). The molar nitrogen to hexosamine ratio in the reprecipitated HC-PP is 50 per cent higher in the product from the older age group than in that from the younger age group, and 100 per cent higher than in the corresponding BN-PP included at the bottom of Table II for comparison.

Multiple extraction procedure. By the single extraction procedure described above, over 20 per cent of the dry weight of the human costal cartilage was extracted and recovered as crude HC-PP. A study was also made to see how much more could be obtained by a series of three successive extractions using the same general extraction procedure. For each experiment about 18 g of dried cartilage was used. The first extraction was made as described above. For the second extraction the cartilage residue at the stage called *R* above was used. To the packed residue (*R*) two volumes of water were added and the mixture was beaten in the VirTis-45 at top speed for one-half hour. Then ethanol was added to bring its concentration to 67 per cent, the mixture was centrifuged at 4,700 rpm for one-half hour, the supernatant solution was filtered through a glass wool plug and a second yield of crude HC-PP was precipitated from the filtrate by addition of potassium acetate. From the packed cartilage residue of this second extraction, a third extraction was made by the same procedure as for the second. In this

TABLE III

Comparison of human costal cartilage and bovine nasal cartilage with respect to the yields of crude PP obtained in each of three successive extractions *

	Human costal cartilage			
Age	HC-PP$_1$	HC-PP$_2$	HC-PP$_3$	Total yield
yrs	*g/g dry cartilage*			
21	0.228	0.105	0.038	0.371
21	0.165	0.112	0.028	0.305
27	0.221	0.110	0.041	0.372
27	0.246	0.100	0.049	0.395
28	0.216	0.101	0.043	0.360
29	0.209	0.083	0.046	0.338
31	0.288	0.099	0.051	0.438
32	0.227	0.087	0.054	0.368
38	0.162	0.060	0.032	0.254
Average	0.218	0.095	0.042	0.355
	Bovine nasal cartilage			
	BN-PP$_1$	BN-PP$_2$	BN-PP$_3$	Total yield
	g/g dry cartilage			
	0.289	0.075	0.020	0.384
	0.231	0.117	0.025	0.373
	0.316	0.076	0.031	0.423
	0.300	0.092	0.025	0.417
Average	0.284	0.090	0.025	0.399

* See first page of text for abbreviations.

way there were obtained three successive yields of crude HC-PP. Table III shows these successive yields in a number of such trials on samples of cartilage from different individuals. For comparison there is also included a similar set of yields from bovine nasal cartilage using the same procedure. It is interesting to note that the spread of values is much higher among the results from human cartilage than among those from bovine cartilage.

RESULTS

Ultracentrifugal fractionation. After finding that the crude BN-PP could be separated into two fractions by ultracentrifugal means (*2*), the same method was tried with the crude HC-PP. Essentially the same procedure was used and need not be described in detail here. In Table IV are shown the essential procedures together with the yields of the products for one particular case. The results resembled those found for crude BN-PP in that two major fractions could be separated which differed in sedimentation characteristics and each of which contained both protein and polysaccharide. They will therefore be distinguished as heavy (PP-H) and light (PP-L) depending upon the ease with which each sediments. As in all other work done with human costal cartilage, this fractionation was less constant in the proportions of PP-H and PP-L obtained with different samples of crude HC-PP than were the proportions obtained from crude BN-PP. Table V shows the yield of a number of such fractionation experiments, each done with a sample of crude HC-PP of a different individual. The crude HC-PP constituting the first extract from the human costal cartilage on fractionation generally yields amounts of PP-L larger than the amounts of PP-H. The ratio of the average PP-L yield to the average PP-H yield is 1.8. This is very different from the corresponding ratio found for the fractionation of crude bovine nasal chondromucoprotein, which is 5.8 (*2*). Thus the crude PP extracted from human costal cartilage differs from that extracted from bovine nasal septa in containing a smaller proportion of the more readily soluble and less readily sedimentable fraction. Another point of interest brought out in the data of Table V is that on applying the ultracentrifugal fractionation procedure to each of the three successive crude HC-PP products extracted from human costal

TABLE IV

Steps in the ultracentrifugal separation of crude HC-PP into the two fractions PP-L and PP-H **

Step material	Procedure	Residue as loose gel	Product† from supernatant
		ml	*g*
1. Crude HC-PP (3.8 g) +	Stir with VirTis-45 at 1,000 rpm 15 min;	108	1.36 PP-L
0.15 M KCl (380 ml)	Centrifuge at 78,000 G 2 hrs		
2. Gel from step 1 (108 ml) +	Stir with VirTis-45 at 1,000 rpm 45 min;		
0.15 M KCl (250 ml)	Centrifuge at 500 G 20 min and discard residue (0.14 g); Centrifuge supernatant solution at 78,000 G 20 min	84	0.80 PP-L
3. Gel from step 2 (84 ml) +	As step 2 (discard residue from centrifuging at 500 G, 0.04 g)	60	0.14 PP-L
0.15 M KCl (350 ml)			
4. Gel from step 3 (60 ml) +	Stir with VirTis-45 at 1,000 rpm 45 min;		
0.15 M KCl (400 ml)	Centrifuge at 500 G 30 min and discard slight residue		0.99 PP-H

* See first page of text for abbreviations.

† In each case the product was precipitated from the supernatant solution by the addition of ethanol (2 vol), centrifuged, washed with ethanol and ethyl ether, and dried *in vacuo*.

cartilage (as in Table III) there is a progressive fall in the ratio of PP-L to PP-H.

Analytical data for a series of samples of PP-L and PP-H prepared from the crude HC-PP from individuals mainly in the age range 20 to 38 years are given in Table VI. To facilitate comparison with previous work done with the analogous products from bovine nasal cartilage, the hexosamine was determined, using galactosamine as a standard. The results for PP-L show a reasonable constancy in the proportion of protein to polysaccharide in products obtained from individuals in the age range 25 to 50 as measured by the molar ratio of nitrogen to galactosamine, while for the few cases in a lower age range the ratio is slightly lower. This ratio, represented by $R_{N/gal}$ is calculated from the determined percentages of nitrogen and galactosamine by the relation:

$$R_{N/gal} = (\%N/14)/(\%\text{galactosamine}/179) = 12.8\ (\%N/\%\text{galactosamine}).$$

The average value of this ratio for HC-PP-L is 2.7. Repeated solution of HC-PP-L in 0.1 M KCl, centrifugation at 78,000 G for 1 hour and reprecipitation with 2 vol of ethanol yielded products with this same composition. In contrast to the relative constancy of $R_{N/gal}$ for HC-PP-L there is for HC-PP-H a wider scatter in this ratio, which averages around 8.5. Direct determination of protein by the biuret method gave 21 per cent protein in PP-L, and 66 per cent protein in PP-H. Both these values are higher than those for the corresponding proteinpolysaccharides of bovine nasal cartilage.

Solutions of HC-PP-L in water are clear enough so that optical rotations can be measured. Determinations of two separate samples gave values for $[\alpha]_D$, 1 per cent in water, $-24.2°$ and $-26.4°$. On the other hand, solutions of HC-PP-H are extremely opalescent.

TABLE V

Yields of PP-L and PP-H from crude HC-PP by the fractionation method of Table IV *

Age	$HC\text{-}PP_1$		$HC\text{-}PP_2$		$HC\text{-}PP_3$	
	PP-L	PP-H	PP-L	PP-H	PP-L	PP-H
yrs	*g./g crude HC-PP*					
0–7	0.520	0.200				
0–5	0.455	0.241				
19–20	0.500	0.290				
21	0.715	0.341				
21	0.675	0.141	0.491	0.248	0.228	0.382
29	0.523	0.339	0.353	0.437	0.200	0.505
31	0.560	0.319				
32	0.605	0.270				
32	0.394	0.478				
36	0.611	0.195				
38	0.496	0.286	0.410	0.465	0.298	0.530
40–50	0.430	0.341				
50	0.470	0.175				
72–99	0.620	0.192				
60–92	0.610	0.320				
Average	0.542	0.305	0.418	0.383	0.242	0.472
PP-L/PP-H	1.78		1.09		0.51	

* See first page of text for abbreviations.

TABLE VI

Nitrogen and galactosamine content of a series of preparations of PP-L and PP-H from human costal cartilage of subjects of various ages *

Age	PP-L			PP-H		
	N	Galactosamine	$R_{N/gal}$	N	Galactosamine	$R_{N/gal}$
yrs	%	%		%	%	
0–5	4.9	29.0	2.1	9.2	18.2	6.5
19–25	5.0	26.2	2.4	9.3	17.1	7.0
21	5.1	28.4	2.3	10.5	16.6	8.1
26–28		28.0		10.1	15.2	8.5
26–28	5.4	25.9	2.6			
28	5.9	27.9	2.8	10.8	14.3	9.6
28	5.9	28.0	2.7			
28	6.3	28.2	2.9	11.1	12.5	11.4
28	5.9	25.8	2.9	11.1	15.3	9.3
29	6.2	28.6	2.8	10.4	15.4	8.7
31	5.6	28.2	2.5	10.2	16.8	7.8
31	5.8	27.2	2.7	8.9	16.2	7.0
36	5.6	25.1	2.8	9.3	17.2	6.9
36	5.8	26.3	2.8	10.4	17.7	7.5
38	5.8	26.2	2.8	11.9	12.8	11.9
50	6.1	25.2	3.0			
Average	5.7	27.1	2.7	10.2	15.8	8.5

* All samples indicated by a single age were from one individual; samples indicated by an age range were made from crude HC-PP pooled from several individuals of ages within the indicated range. See first page of text for abbreviations.

DISCUSSION

The work presented shows that the methods developed for the isolation of the proteinpolysaccharides from bovine nasal septa are applicable to human costal cartilage and give results roughly parallel but differing in detail. A crude HC-PP analogous to crude BN-PP (earlier called chondromucoprotein) can be isolated in somewhat lower yields and with a generally higher protein content and also a more variable composition with regard to the proportion of protein and polysaccharide (Table I). The crude HC-PP also differs from the crude BN-PP in that after a single

reprecipitation its composition is changed, particularly with material from persons over 30 years old, in the direction of a decreased proportion of protein to polysaccharide. On further reprecipitation there is no significant further change in composition (Table II). The total amount of such crude proteinpolysaccharide that can be isolated by a series of three successive extractions from a sample of human costal cartilage amounts to 35 per cent of the dry weight of the cartilage, while a similar triple extraction of bovine nasal cartilage yields a total of 40 per cent of its dry weight (Table III).

The method developed for the ultracentrifugal fractionation of crude BN-PP is also applicable to crude HC-PP. Two clear-cut fractions can be obtained, an easily sedimentable fraction (HC-PP-H) and a less readily sedimentable fraction (HC-PP-L). The yields of each of these fractions varies rather widely with the individual from whom the crude chondromucoprotein was obtained (Table V). Just as the crude HC-PP has a higher protein content than BN-PP, so both HC-PP-L and HC-PP-H have higher protein contents than their bovine analogs BN-PP-L and BN-PP-H (Table VI).

In the case of bovine nasal cartilage it was shown that, on treatment with alkali, both PP-L and PP-H yield chondroitin sulfate with the same optical rotation, $[\alpha]_D = -24°$. With the products from human costal cartilage, greater difficulty was encountered in this as in all other procedures and the results cannot yet be reported. Work of Meyer, Hoffman and Linker (3) and Kaplan and Meyer (4) indicates that both chondroitin sulfates A and C and keratosulfate are found in human costal cartilage. It is now of importance to find which of these is in the PP-L or PP-H and which remains in the cartilage residue, for this residue contains about one-half of the hexosamine of the original human costal cartilage. The amount of hexosamine left in the bovine nasal cartilage residue after extraction of the crude BN-PP is only about 20 per cent of that originally present.

With the successive improvements reported in the isolation and fractionation of the proteinpolysaccharides of cartilage, it begins to be feasible to attack a problem of concern in the rheumatic diseases: the amount and condition of the proteinpolysaccharides in cartilages, particularly the articular cartilage, of rheumatic patients.

SUMMARY

Using the extraction procedure developed for use with bovine nasal cartilage, a study of human costal cartilage has been made. By three successive extractions with water, crude proteinpolysaccharide can be isolated in amounts of 20 to 35 per cent of the dry weight of the cartilage. While similar in properties to the crude proteinpolysaccharide reported from bovine nasal cartilage, the corresponding product from human costal cartilage contains more protein and less polysaccharide. Ultracentrifugal fractionation at 78,000 × G resulted in separation of this crude product into a rapidly sedimenting fraction and a nonsedimenting fraction. Yields and analytical data on these products are given. Both fractions contain more protein than do the corresponding fractions made from bovine nasal cartilage.

REFERENCES

1. Malawista, I., and Schubert, M. Chondromucoprotein; new extraction method and alkaline degradation. J. biol. Chem. 1958, **230**, 535.
2. Gerber, B. R., Franklin, E. C., and Schubert, M. Ultracentrifugal fractionation of chondromucoprotein. J. biol. Chem. In press.
3. Meyer, K., Hoffman, P., and Linker, A. Mucopolysaccharides of costal cartilage. Science 1958, **128**, 896.
4. Kaplan, D., and Meyer, K. Ageing of human cartilage. Nature (Lond.) **183**, 1267.

THE EFFECT OF SODIUM DILANTIN ADMINISTRATION UPON THE CHEMISTY OF THE SKIN *

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The gingival hyperplasia associated with Dilantin therapy has been reviewed by Ziskin, Stowe and Zegarelli (1). Although a similar pathologic change could be induced in the ferret (2), it has been impossible to produce this condition in rats (3). However, Shafer, Beatty and Davis (4) have demonstrated that Dilantin-treated rats show a marked increase in the tensile strength of their healing wounds. These workers attributed this elevation in the tensile strength of wounds with parenteral Dilantin therapy to an increase in collagen synthesis, the same mechanism underlying the gingival hyperplasia occurring in young epileptics under Dilantin therapy. Forscher and Cecil (5) were unable to find any significant differences in the hydroxyproline content of healing palate tissue between control and Dilantin-treated rats.

This paper describes the effects of massive daily intraperitoneal injections of Dilantin Sodium upon the distribution of hydroxyproline, nitrogen and hexosamine within the connective tissue (skin) of the rat, as determined by using a sequential extraction technique to effect chemical dissection of the tissue.

MATERIAL AND METHODS

Thirty male Sprague-Dawley rats (210 to 230 g) were subjected to daily intraperitoneal injection of 1 ml of isotonic saline containing a suspension of 25 mg per ml of Dilantin Sodium.[1] At 2, 5, 7, 11 and 16 days after initiation of the experiment, groups of 6 rats each were sacrificed by either anesthesia or exsanguination. Six g samples of abdominal rat skin were collected from each animal, carefully shaven and dissected clean of adhering fat, fascia and muscle and minced with scissors. The tissues were then stored frozen until the completion of the experiment. Samples of each skin (300 mg) were then hydrolyzed for 8 hours at 100° C with 10 ml of 4 N HCl per g of skin. The sera were diluted 1:1 with 8 N HCl and hydrolyzed in similar fashion. The acid hydrolysate was analyzed for total hexosamine (6), hydroxyproline (7) and nitrogen, as has been described previously (8).

Hydroxyproline, as a signal amino acid of collagen, is an excellent indicator of this protein (9). Hexosamine may be found in both ground substance mucopolysaccharides and serum glycoproteins contaminating the matrix of the skin (10). Nitrogen is a standard measure of total protein.

Duplicate 5 g samples of rat skin were extracted with 50 ml of 0.15 M NaCl in the cold (3° C) using a Lourdes high-speed tissue blender for 45 minutes. After extraction, the mixtures were stored for 18 hours at 4° C.

After centrifugation at 25,000 × G and 4° C, the clear supernatant was removed. The insoluble residue was then extracted similarly with 0.5 M NaCl and after standing for 18 hours in the cold, the residue after centrifugation was in turn re-extracted with 100 ml of 0.5 M citrate buffer (pH 3.6). These conditions for extraction are such as to solubilize about 75 to 95 per cent of all the material exhaustively extracted by each solvent.

These fractions were mixed 2:1 with 12 N HCl and hydrolyzed as described above for hexosamine, hydroxyproline and nitrogen analysis.

Since cortisone administration does not increase the dermal content of either hexosamine or collagen (11), and since we were unable to measure any increase of these components in the skin of rats stressed by intraperitoneal injection (unpublished data), untreated animals of weights similar to those of the experimental animals were used as a control.

Statistical evaluation of the data presented below indicated that means which differ by more than 15 per cent are significantly different ($p = 0.05$). This level of significance, i.e., differences of more than 15 per cent are significant, obtains for all the results described in all of the tables.

EXPERIMENTAL AND RESULTS

The results of the analysis of rat skin for hydroxyproline, hexosamine and nitrogen after various numbers of daily intraperitoneal injections of 25 mg of Dilantin Sodium were expressed per gram of stored, cleaned and shaven tissue and are presented in Table I.

* Supported in part by Grant-in-Aid A-3899 from the National Institute of Arthritis and Metabolic Diseases, Bethesda, Md.

[1] Parke, Davis (sodium 5,5-diphenylhydantoinate).

After four doses of Dilantin there is a significant increase in dermal hydroxyproline. This increase becomes maximal after 11 injections of drug with the treated rat skin containing about 150 μmoles more of hydroxyproline per g of skin than does the untreated animal. Hexosamine increased by about 4 μmoles per g during this period, while the nitrogen content increased by 6 mmoles per g.

The chemical analyses described above were based upon the weight of cleaned and shaven rat skin which had been stored in the frozen state from 4 to 21 days. During the first four days all tissues lost about 30 per cent of their weight through sublimation of dermal water. With further storage in the deep freeze less than a few milligrams of water were lost per gram per day. For this reason the values reported in Table I and below must be corrected for the loss of 300 mg of water per g if the results are to be expressed per gram of *fresh* cleaned and shaven skin. Because of the extraordinary constancy of this 30 per cent loss of weight via water sublimation, we prefer to report most of our data per gram of *stored* cleaned and shaven skin.

The absolute dermal content of water from normal rats and animals subjected to 7 and 11 doses of Dilantin was determined by lyophilization. The concentration of dermal collagen may be calculated from the finding that pure collagen contains about 13.6 per cent hydroxyproline, or 1 μmole per g (12). The amount of noncollagenous protein may be calculated from the fact that collagen contains about 18.5 per cent nitrogen or 13.3 μmoles per mg (12) and most other proteins contain about 16 per cent or 11.4 μmoles of nitrogen per mg. Multiplication of the micromoles of hydroxyproline, or milligrams of collagen, per gram of skin times 13.3 results in the micromoles of collagen nitrogen, and the difference between this value and the value for the total dermal nitrogen divided by 11.4 results in the milligrams of noncollagenous protein. The difference between the sum of the weight of water, collagen, and noncollagenous proteins, in milligrams per gram of tissue, and 1,000 represents the milligrams of fat in the skin. The results of these calculations are shown in terms of *fresh* shaven and cleaned skin in Table II. Correction of the values per gram of *stored* tissue reported in Table I for water loss results in essentially identical results.

TABLE I

The analysis of whole rat skin after Dilantin administration *

No., daily inject.	Hypro	Hex	N
	μmoles/g	*μmoles/g*	*mmoles/g*
0	300 ± 27	11.0 ± 0.8	6.1 ± 0.5
2	330 ± 31	14.7 ± 0.9†	9.0 ± 0.6†
4	349 ± 30	14.6 ± 1.0	10.7 ± 0.6
7	366 ± 28	15.2 ± 1.1	10.5 ± 0.7
11	468 ± 35	15.7 ± 1.0	12.4 ± 0.8
16	447 ± 35	15.1 ± 1.1	12.5 ± 0.7

* Mean values of duplicate extracts analyzed in triplicate Hypro = hydroxyproline, hex = hexosamine, N = nitrogen.

† All values below the line are significantly increased above normal ($p < 0.05$).

TABLE II

The chemical anatomy of fresh rat skin before and after various doses of Dilantin

Component	Normal	After 7 doses	After 11 doses
	mg/g	*mg/g*	*mg/g*
Water*	550	300	300
Collagen	200	245	300
Noncollagenous protein	122	395	382
Fat†	128	60	18

* By lyophilization.

† By difference.

The fat content of normal skin as calculated by difference was 128 mg per g. Kao, Schwartz, Treadwell and McGavach (13) recently reported the fat content of the skin of rats of equivalent weight to be about 125 mg of lipid per g of fresh skin.

The effects of Dilantin administration upon the hydroxyproline, hexosamine and nitrogen content per milliliter of rat sera after various numbers of Dilantin injections are shown in Table III.

These data indicate that there are statistically significant decreases in serum hydroxyproline and hexosamine and significant increases in serum nitrogen. None of these changes is as marked as those found in the skin.

In an attempt to determine the nature of the changes in dermal chemistry with Dilantin administration, the skin samples were extracted sequentially with 0.15 and 0.5 M sodium chloride as

TABLE III

The analysis of rat serum after Dilantin administration *

No., daily inject.	Hypro	Hex	N
	μmoles/g	*μmoles/g*	*mmoles/g*
0	0.70 ± 0.05	3.3 ± 0.2	0.68 ± 0.05
	†	†	†
2	0.45 ± 0.03	1.7 ± 0.1	0.80 ± 0.06
4	0.33 ± 0.02	1.1 ± 0.1	0.81 ± 0.06
7	0.40 ± 0.03	1.3 ± 0.1	0.86 ± 0.06
11	0.52 ± 0.03	1.2 ± 0.1	0.88 ± 0.06
16	0.40 ± 0.04	1.1 ± 0.1	0.88 ± 0.07

* Mean values of triplicate analyses of sera from six animals.

† All values below the line are significantly different from normal ($p < 0.05$).

described above. Microscopically, the cellular material was intact only after isotonic saline extraction, and the electron microscope indicated that the collagen was morphologically intact after both saline extractions. The amount of hydroxyproline, hexosamine and nitrogen extracted per gram of skin by these solvents is shown in Table IV.

The quantity of material soluble in isotonic saline, although occasionally differing significantly from the untreated normal, did not show any real pattern of response to Dilantin administration. The amount of hydroxyproline soluble in 0.5 M saline decreased significantly from normal with the second dose of Dilantin, while the hexosamine soluble in this fraction increased significantly with drug administration. The decreases in 0.5 M saline-soluble nitrogen with Dilantin were only occasionally statistically significant.

More meaningful changes in the quantity of material extractable from the skin of Dilantin-treated rats were found in the analysis of the citrate buffer extracts shown in Table V.

There was an immediate increase of 18 μmoles per g of hydroxyproline with Dilantin administration. The increase of 150 per cent with respect to hydroxyproline was paralleled by a 100 per cent increase in soluble nitrogen. Hexosamine was increased maximally (700 per cent) after four doses of Dilantin, suggesting that the syntheses of hexosamine and collagen are independent.

There was a slight increase in the ratio of hydroxyproline to nitrogen in this extract which may indicate a greater purity of the acid-soluble collagen produced in response to Dilantin administration.

By summation of all three soluble fractions, the total soluble material of the skin may be calculated. Subtraction of this value from the total skin content, described in Table I, permits the calculation of the insoluble dermal components, as shown in Table VI.

Initially, there was an increase in soluble dermal hydroxyproline, or collagen, with Dilantin administration. The amount of soluble nitrogen was significantly increased under these conditions. The soluble hexosamine increased about 30 per cent with Dilantin. The ratio of soluble hydroxyproline and hexosamine to nitrogen remained essentially constant following the daily administration of this drug. These ratios did not differ from those obtained from normal untreated rat skin.

The insoluble hydroxyproline increased significantly above normal after the fourth dosage of Dilantin. After the eleventh dose of the drug, there was a 60 per cent increase in insoluble hy-

TABLE IV

Analysis of the saline-soluble component of rat skin after Dilantin administration

No., daily inject.	Extracts					
	0.15 M NaCl			0.50 M NaCl		
	Hypro	Hex	N	Hypro	Hex	N
0	7.7	5.1	1.30	29	2.4	1.20
2	11.1*	5.7	1.39	21*	3.0*	0.95*
4	8.9*	6.5*	1.33	17*	3.4*	1.10
7	6.1	6.3*	1.43	23*	2.9*	1.10
11	9.0*	6.4*	1.17	20*	3.5*	0.93*
16	6.5	5.9*	1.11*	15*	3.4*	0.95*

* Significantly different from normal ($p < 0.05$).

TABLE V

Analysis of the citrate-soluble components of rat skin after Dilantin administration

No., daily inject.	Hypro	Hex	N
	μmoles/g	*μmoles/g*	*mmoles/g*
0	12.0 ± 0.8	0.10 ± 0.1	0.60 ± 0.05
	*	*	*
2	30.7 ± 2.0	0.45 ± 0.3	1.27 ± 0.10
4	30.1 ± 2.0	0.70 ± 0.7	1.30 ± 0.10
7	30.0 ± 2.0	0.70 ± 0.6	1.28 ± 0.10
11	28.5 ± 2.0	0.65 ± 0.5	1.14 ± 0.13
16	30.0 ± 2.0	0.76 ± 0.5	1.28 ± 0.10

* All means below the line are significantly elevated above normal ($p < 0.05$).

TABLE VI

The total soluble and insoluble component of rat skin after Dilantin administration

No. daily inject.	Total soluble			Insoluble		
	Hypro	Hex	N	Hypro	Hex	N
0	48.7	7.6	3.1	251	3.4	3.0
2	62.6*	9.2*	3.6	267	5.5*	5.4*
4	56.0	10.6*	3.7*	293*	4.0	7.0*
7	59.1*	9.8*	3.8*	307*	5.4*	6.7*
11	57.5*	10.5*	3.2	410*	5.2*	9.2*
16	50.0	10.1*	3.1	397*	5.0*	9.4*

* Significantly elevated above normal ($p < 0.05$).

droxyproline. This profound increment in collagen was not paralleled with a similar increase in insoluble hexosamine; this material increased maximally (50 per cent) after only two injections of Dilantin. The concentration of insoluble nitrogen doubled after the fourth Dilantin injection, and tripled after the eleventh injection. The increase in the total dermal nitrogen of 6 mmoles per g of skin with Dilantin, as shown in Table I, seemed to be associated almost entirely with the insoluble components of the skin. Similarly, most of the increase of 150 μmoles of hydroxyproline per g of tissue was also found in the insoluble fraction. The ratio of hydroxyproline to nitrogen of very pure tendon collagen and soluble collagen is about 84 (μmoles per mmole), while a similar ratio of the insoluble residue from normal skin was about 83. With Dilantin administration, the ratio of hydroxyproline to nitrogen decreased to about 42, evidence of a disproportionate increase in insoluble noncollagenous protein.

DISCUSSION

From the data reported above, it may be concluded that Dilantin produces four major alterations in dermal chemistry: *1*) a marked decrease in tissue water from 55 to 30 per cent and in dermal fat from 12.5 to 0.2 per cent; *2*) a marked increase in collagen (50 per cent); *3*) a marked increase in hexosamine (37 per cent); *4*) a marked increase in a noncollagenous protein.

That these changes are not due to contamination of the tissues with blood elements is indicated by the minor changes in the hexosamine, hydroxyproline and nitrogen content of the sera of these animals. The profound increase of these components in the skin is associated with a quantitatively minor decrease of hydroxyproline and hexosamine in the sera.

The isotonic saline-soluble components of the skin, mostly mucopolysaccharides, serum proteins, glycoproteins and so forth, are not meaningfully altered with Dilantin administration. The most important change in the 0.5 M saline-soluble materials is a significant decrease in the hydroxyproline content of this fraction, which is believed to extract the precursor of collagen (14) as well as glycoproteins and other proteins which are strongly bound to the insoluble components of the skin.

Citrate buffer (pH 3.6) soluble material, an extractant which removes reticulin and/or *"procollagen"* (14), shows a marked increase (150 per cent) in soluble collagen with Dilantin administration. The parallel increase in acid-soluble nitrogen (100 per cent) associated with this hydroxyproline increment serves to maintain a constant ratio of hydroxyproline to nitrogen (micromoles per millimoles) of 24. The constancy of this ratio indicates that the increases in acid-soluble nitrogen are approximately proportional to the increases in acid-soluble collagen with Dilantin administration.

The essential independence of the collagen and hexosamine synthesizing processes is suggested by the fact that the increase in acid-soluble hexosamine does not parallel the increments in nitrogen or hydroxyproline with respect to time. This hexosamine-containing moiety produced in response to Dilantin is not a glycoprotein, since the contribution of nitrogen from this compound would have altered the hydroxyproline-nitrogen ratios considerably. It cannot be a *free* mucopolysaccharide, since it is not soluble in either saline extract. This material is most probably a mucopolysaccharide, tightly bound to the acid-soluble proteins by bonds of higher energy than those that can be destroyed by high ionic strength solvents.

The calculation of the concentration of insoluble material in the skin of rats treated with Dilantin indicates that there is a profound increase in insoluble collagen (58 per cent), insoluble hexosamine and nitrogen. Most of the 150 mg increase in dermal collagen with Dilantin administration described in Table I was found in the insoluble residue. Similarly, most of the 6 mmole increase in dermal nitrogen with drug dosage is insoluble. The increase in dermal hexosamine (4 μmoles) is

evenly divided between the total soluble and insoluble fractions. This latter component in the insoluble fraction is maximally elevated after two doses of Dilantin, while the hydroxyproline and nitrogen require about 11 doses of the drug to effect maximal increment.

The nature of this insoluble hexosamine-containing component may well be a mucopolysaccharide strongly bound to either the insoluble collagen or the other insoluble proteins described below, and as such might bear a relationship to the mucopolysaccharide soluble only in citrate buffer.

The ratio of hydroxyproline to nitrogen (micromoles per millimoles) of the insoluble residue from the untreated animals was similar to that of pure collagen (i.e., 83). With Dilantin administration, this ratio decreased to about 45, indicating that the increase in insoluble nitrogen was far greater than the increase in insoluble dermal collagen. One of the major alterations in the chemistry of the skin in response to Dilantin is therefore the appearance of a unique insoluble noncollagenous protein. That this material is probably not a glycoprotein is indicated by the variation of the hexosamine to nitrogen ratios with increasing Dilantin dosage as calculated from Table VI.

SUMMARY

Dilantin administration results in the following changes in the chemistry of rat skin:

1. A marked decrease in dermal water and fat.
2. A marked increase in acid-soluble collagen and acid-soluble mucopolysaccharide.
3. A marked increase in insoluble collagen and hexosamine.
4. The appearance of a unique insoluble noncollagenous protein.

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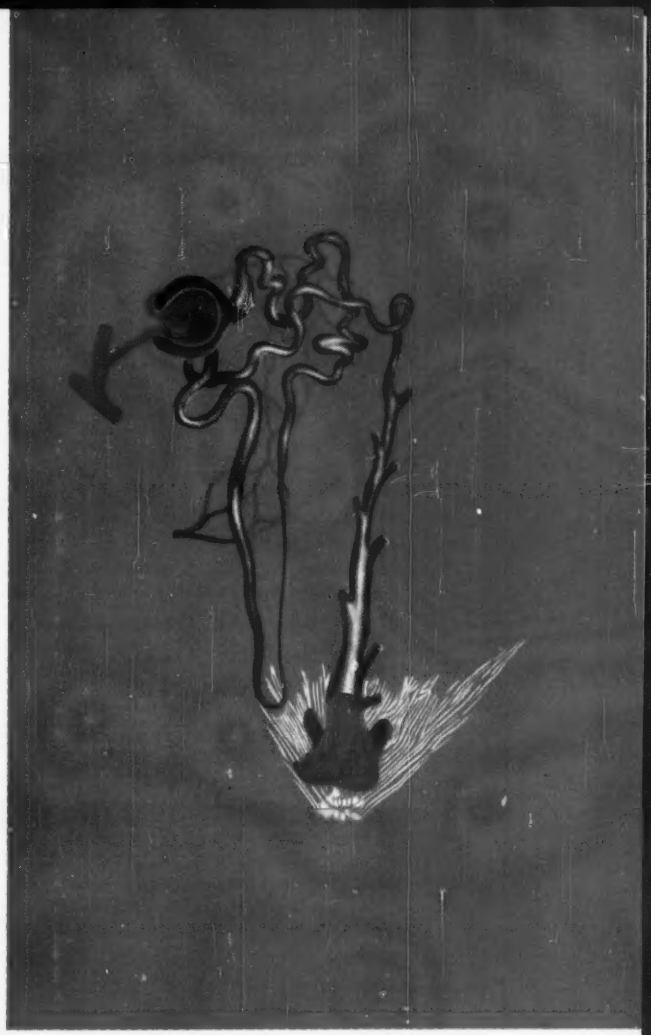
REFERENCES

1. Ziskin, D. E., Stowe, L. R., and Zegarelli, E. V. Dilantin hyperplastic gingivitis: Its cause and treatment; differential appraisal. Amer. J. Orthodont. 1941, **27**, 350.
2. King, J. D., and Gimson, A. P. Experimental investigations of paradontal disease in the ferret and related lesions in man. Brit. dent. J. 1947, **83**, 148.
3. Swenson, H. M. A failure to produce enlargement of gingiva in rats by using Dilantin Sodium. J. dent. Res. 1954, **33**, 468.
4. Shafer, W. G., Beatty, R. E., and Davis, W. B. Effect of Dilantin Sodium on tensile strength of healing wounds. Proc. Soc. exp. Biol. (N. Y.) 1958, **98**, 348.
5. Forscher, B. F., and Cecil, H. C. Biochemical studies on acute inflammation. II. The effect of Dilantin. J. dent. Res. 1957, **36**, 927.
6. Blix, G. The determination of hexosamines according to Elson and Morgan. Acta chem. scand. 1948, **2**, 467.
7. Martin, C. J., and Axelrod, A. E. A modified method for the determination of hydroxyproline. Proc. Soc. exp. Biol. (N. Y.) 1953, **83**, 461.
8. Houck, J. C., and Jacob, R. A. The dermal collagen response to local inflammation. J. biol. Chem. 1961. Submitted for publication.
9. Neuman, R. E., and Logan, M. A. The determination of collagen and elastin in tissues. J. biol. Chem. 1950, **186**, 549.
10. Boas, N. F. Distribution of hexosamine in electrophoretically separated extracts of rat connective tissue. Arch. Biochem. 1955, **57**, 367.
11. Sobel, H., Gabay, S., and Johnson, C. Effect of cortisone on connective tissues of the rat. Proc. Soc. exp. Biol. (N. Y.) 1958, **99**, 296.
12. Gustavson, K. H. The Chemistry and Reactivity of Collagen. New York, Academic Press, 1956.
13. Kao, K. Y., Schwartz, J., Treadwell, C., and McGavach, T. Effect of age and sex upon lipid composition of tissues of rat. Proc. Soc. exp. Biol. (N.Y.) 1960, **103**, 666.
14. Jackson, D. S. The formation and breakdown of connective tissue *in* Connective Tissue Symposium, R. E. Tunbridge, Ed. Oxford, Blackwell Scientific Publications, 1957, p. 62.



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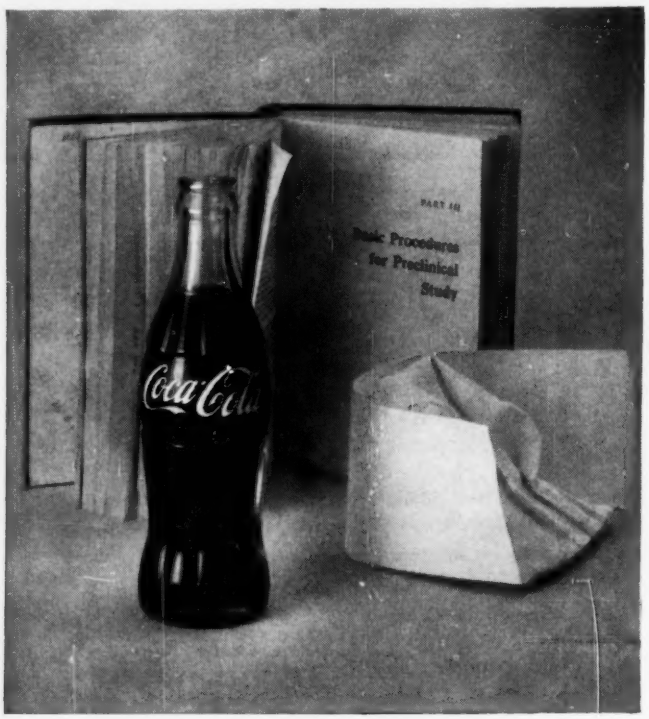

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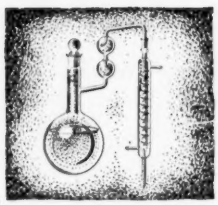

IN NOVEMBER – (1910) – there is reported[1] a study of the influence of alcohol upon nitrogen metabolism in dogs and man. Moderate doses of alcohol are found to exert a protein-sparing action; with larger quantities there is some nitrogen loss. Perhaps the most significant impression is the absence of any *profound* disturbance in protein metabolism, even when comparatively large doses are continued for days and weeks.

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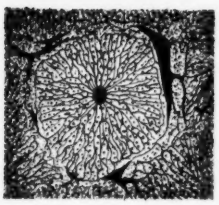

IN NOVEMBER – (1939) – a letter from the Coris[2] discusses certain apparent differences between liver, brain, and muscle phosphorylase. The liver enzyme converts glucose-1-phosphate to glycogen more rapidly than do preparations from other tissues. This is attributable to contamination of liver phosphorylase by glycogen, rather than to an intrinsic difference in the enzyme itself. This established the role of glycogen as a primer in glycogenesis.

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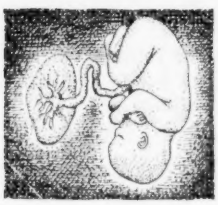

IN NOVEMBER – (1952) – a report from Scandinavia discusses the determination of the nucleic acid content of human placenta. There is good agreement between results obtained by Hammarsten's method and by application of cysteine reactions to the hot T.C.A. extract. Schmidt and Thannhauser values showed considerable divergencies. P.N.A. content decreases sharply with the aging of the placenta, while D.N.A. increases slightly. Thus, there is marked decrease in the P.N.A./D.N.A. ratio during pregnancy.[3]

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1. Mendel, L. B., and Hilditch, W. W.: The influence of alcohol upon nitrogenous metabolism in men and animals. Am. J. Physiol. *27*:1 (Nov.) 1910. 2. Cori, G. T., and Cori, C. F.: Letters to the Editor: The activating effect of glycogen on the enzymatic synthesis of glycogen from glucose-1-phosphate. J. Biol. Chem. *131*:397 (Nov.) 1939. 3. Brody, S.: Quantitative studies on the nucleic acids in human placenta. Acta Chem. Scandinav. *7*:507,1953.

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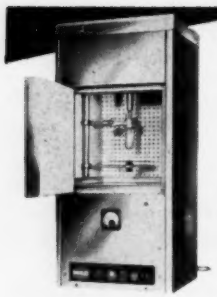

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Vol. V, No. 8

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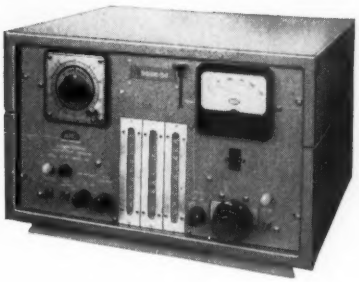

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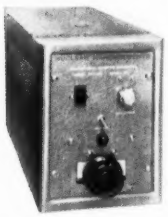

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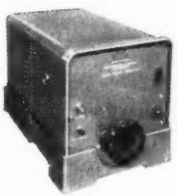

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